The Developmental Potential of Embryos and cells that are Deficient in Glycolysis

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Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree nor is any part of it being submitted concurrently in candidature for another degree. All experiments were performed at the Department of Obstetrics and Gynaecology, University of Edinburgh unless otherwise stated.

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ABSTRACT

Embryos homozygous for a null allele of the gene that encodes the glycolytic enzyme, glucose phosphate isomerase (GPI), were found to die shortly after implantation (West *et al*, 1990, Merkle & Pretsch, 1992). Although the homozygous GPI null embryos cannot produce their own GPI, because they lack the appropriate gene, they survived until 7.5 - 8.5d.p.c. (West *et al*, 1990) and certain tissues survived until 10.5d.p.c. A histological study was undertaken to determine when the first signs of abnormality become apparent in the dying homozygous GPI null embryos. The critical time period for these mutant embryos was found to be between 6.5d.p.c. and 7.5d.p.c., shortly after the oocyte coded GPI is exhausted and the embryo has to rely on its own production of the enzyme. The embryos fail to gastrulate properly and produce only a small amount of mesoderm. The abnormally developed egg cylinder expands to form an empty sac like structure. The membrane that resembles the yolk sac is infact comprised of extraembryonic ectoderm and extraembryonic endoderm.

Aggregation chimaeras were produced between homozygous GPI null embryos and normal embryos to examine whether homozygous GPI null cells could survive for longer when combined with normal cells. Because the homozygous GPI null embryos are embryo lethal, two heterozygotes were intercrossed to produce embryos, 25% of which should be homozygous for the null alllele. All of the embryos produced from intercrossing the two heterozygotes were aggregated to normal 8-cell embryos. The chimaeras were analysed at 12.5d.p.c. Several markers were used to analyse the resultant chimaeras, the two components of the chimaera vary at the *Gpi-1s locus* and therefore differences in their electrophoretic mobilities are apparent after GPI electrophoresis. One component of the chimaera is pigmented, the other albino (hybrid strain CF1), therefore the proportions of the pigmented to non-pigmented cells can be determined. Two estimates of eye pigmentation were made, one at the time of dissection and one from histological sections. The pigmented component of the chimaera carries a mouse β-globin transgenic marker inserted on chromosome 3.

It was found that homozygous GPI null cells could be rescued and survive until beyond 12.5d.p.c. in the various tissues analysed (brain, tail, amnion, yolk sac mesoderm, yolk sac endoderm, and placenta). A further chimaera experiment was undertaken to determine whether homozygous GPI null chimaeras could develop to term. Eleven chimaeras were produced in this experiment, including one female which was identified as a homozygous GPI null chimaera. Breeding data from this female reinforced tha fact that she was a null and showed that the trait could be passed through the germ line.

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List of Abbreviations

BSA	Bovine Serum albumin
d.	Days
DDSA	Dodecenyl Succinic Anhydride
DIG	Digoxygenin
DMP30	(Dimethylaminomethyl) Phenol
F6P	Fructose-6-Phosphate
G6P	Glucose-6-Phosphate
GPI	Glucose Phosphate Isomerase
HRP	Horse Radish Peroxidase
NK	Natural Killer Cell
OD	Optical Density
p.c.	*post coitum*
PLP	Poly L-lysine Paraformaldehyde
PMN	Polymorphonuclear Leucocytes
TCA	Tricarboxylic Acid Cycle
TESPA	3, Aminopropyltriethoxysilane
Tg	Transgene
TGF-β	Transforming Growth Factor β
TNF-α	Tumour Necrosis Factor α
TPI	Triose Phosphate Isomerase
X Phos	5-Bromo-4-chloro-3-indolyl-phosphate

CHAPTER 1

INTRODUCTION

1.1 EMBRYONIC DEVELOPMENT IN THE MOUSE

Fetal gestation takes between 19 and 21 days in the mouse, depending on the strain. During this time the fertilized egg undergoes a period of pre- and postimplantation development. At fertilization, the oocyte and spermatozoa come together in the ampullary region of the oviduct. Once fertilized, the egg travels down the oviduct into the uterus, undergoing several cell divisions in which the embryo does not increase in size but the cell number increases. At the 8 - 16 cell stage, the embryo compacts to form a morula and individual cells can no longer be distinguished. Cavitation then occurs and a blastocyst forms, which is now composed of an inner cell mass (ICM) which will give rise to the embryo and the trophectoderm which will contribute to the extraembryonic tissues of the conceptus.

The trophectoderm becomes organized into an epithelium and becomes specialized into 2 subpopulations of cells, the mural and polar trophectoderm (Hogan *et al*, 1986). The mural trophectoderm is derived from the cells around the blastocoel cavity which are not in contact with the ICM. These cease to divide and become large and polytene and are the primary trophoblast giant cells. The polar trophectoderm cells consist of those cells in contact with the ICM. These continue to proliferate, although some migrate around the embryo to replace the primary trophoblast giant cells and become polytene themselves. A finger like projection of polar trophectoderm penetrates into the blastocoel to form the extraembryonic endoderm and pushes the ICM derivatives ahead of it. A proportion of polar trophectoderm cells also penetrate into the endometrium to form the placenta. Some

of these cells and cells of the chorion become polytene and become secondary giant cells.

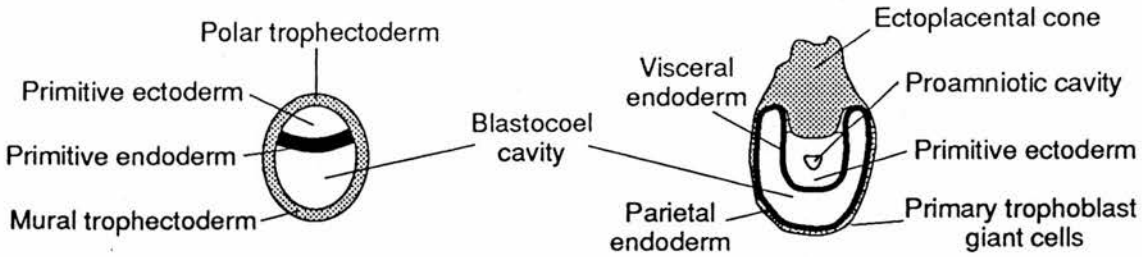
Differentiation of the ICM begins at approximately 4.0 d.p.c., shortly before implantation. The ICM becomes organized into a layer of primitive ectoderm which gives rise to the ectodermal, mesodermal and endodermal tissues of the fetus, some extraembryonic tissues, the amnion, yolk sac mesoderm, allantois and mesoderm of the chorion.

The primitive endoderm gives rise to two cell types, the parietal and visceral endoderm, both of which are extraembryonic. The parietal endoderm is a single layer of cells, which secretes an acellular basement membrane (Reichert's membrane), between the parietal endoderm cells and the trophoblast. The visceral endoderm gives rise to the visceral endoderm of the yolk sac.

At about 4.5 - 5d.p.c the blastocyst hatches from the zona pellucida and implants in the uterine wall. By this stage three primary developmental lineages have been established: the trophectoderm, the primitive endoderm (also known as the hypoblast) and the primitive ectoderm (also known as the epiblast). The entire fetus is derived from the primitive ectoderm lineage.

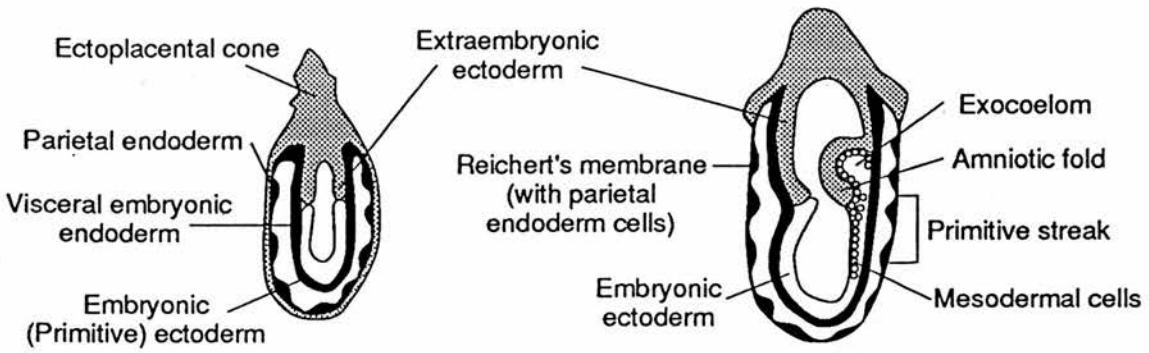
The process during which the three definitive germ layers of the embryo, the ectoderm, mesoderm, and endoderm are formed is gastrulation. The primitive streak forms as a thickening at the junction of the embryonic ectoderm and endoderm and extends anteriorly at approximately 6.5 - 7.0 d.p.c. (Fig 1.1). The formation of primitive streak marks the beginning of gastrulation and defines the anteroposterior axis of the embryo. Mesoderm forms by ingression of the epiblast in the area of the primitive streak. The process of gastrulation is highly complex and as yet not completely understood. However by studying the passage of labelled cells through the primitive streak at different levels, the fate of these cells can be mapped and the

Fig 1.1 Diagram of early postimplantation development of the mouse redrawn from Hogan *et al*, 1986. Different lineages are shaded according to their developmental lineage, primitive ectoderm is unshaded, the primitive endoderm is black and the trophectoderm lineage is grey.



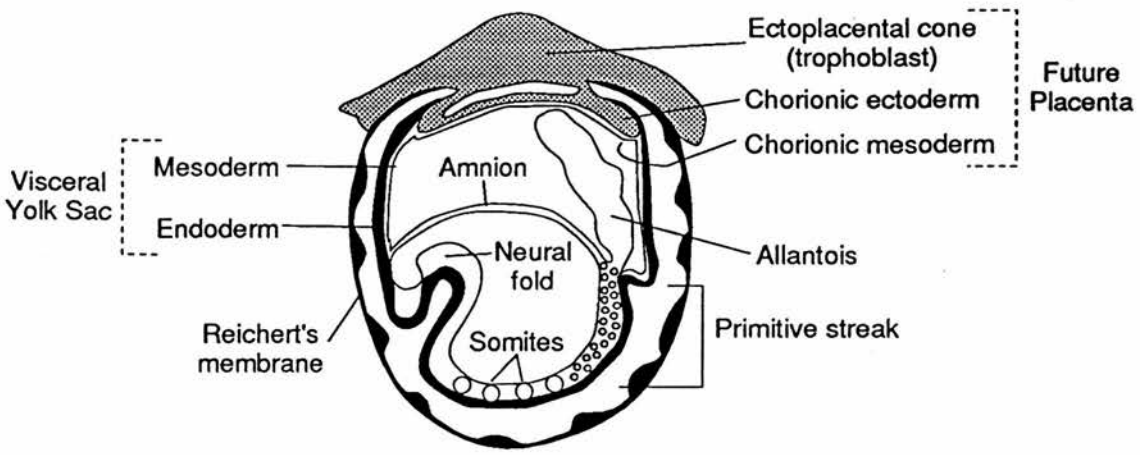
4.5 d.p.c

5.5 d.p.c



6 d.p.c

Early primitive streak ~ 7 d.p.c.



8.5 d.p.c.

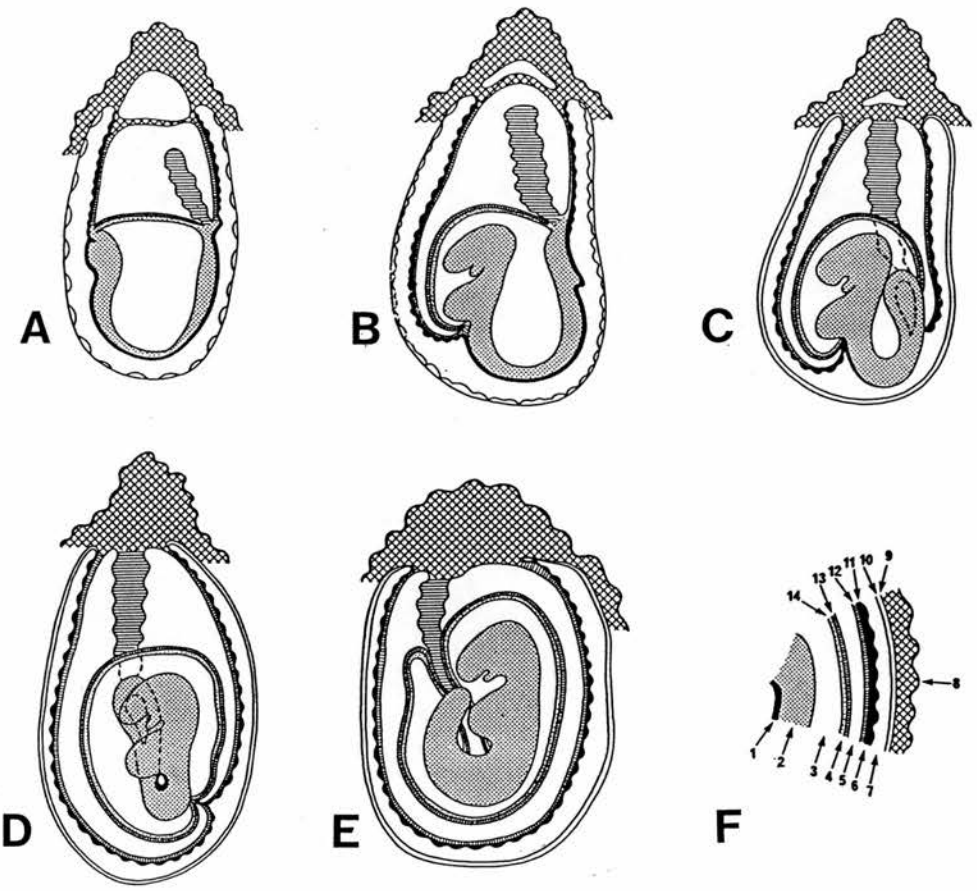
Primitive ectoderm (epiblast) derivative Primitive endoderm Trophectoderm

tissues that certain areas of the streak give rise to can be determined. Using tritiated thymidine to label cells which were then grafted into areas of the embryonic ectoderm, Tam and Beddington (1987) demonstrated that in 8.5d embryos, the posterior streak gave rise to extraembryonic mesoderm, the middle portion gave rise to lateral mesoderm and the anterior region generated mainly paraxial mesoderm, gut and notochord. Lawson *et al* (1991) injected individual cells labelled with horseradish peroxidase (HRP) into younger streak stage embryos (6.7d) and followed their development through one day of culture. They found that the most anteriorly positioned mesoderm cells originated posteriorly and had passed through the streak very early and migrated away. Cells in the posterior part of the streak originated from anterior cells of the epiblast and the cells of the head process were derived from cells in the anterior part of the streak. Endoderm cells were derived from an area similar to the head processes and the yolk sac and amnion mesoderm developed from the posterolateral and posterior epiblast. This study also demonstrated that descendants of the marked cells were not always confined to a single germ layer. By 7.5 days, the three germ layers have formed and the embryo displays the three cavities characteristic of a late egg cylinder, the amniotic, the exocoelomic and the ectoplacental cavity.

The early somite stage embryo is U-shaped (Fig 1.1) and initially the germ layers are inverted, with the endoderm on the outside and the ectoderm inside. The whole conceptus undergoes a 180^{o} rotation anticlockwise about the midpoint of the U. These movements are responsible for the complete envelopment of the embryo by its membranes (Fig 1.2). Formation of the placenta occurs at about 8.5 - 9.0 days and the connection between the fetal and maternal circulation is formed. The fetus then undergoes a period of organogenesis, forming the body systems which will allow the pup to survive at birth.

Fig 1.2 Taken from M. Kaufman, 1990, in Postimplantation Mammalian Embryos: A Practical Approach, Ed. A. Copp and D. Cockcroft.
Simplified diagrammatic sequence to illustrate changes in the conformation of the mouse embryo and the way in which the extraembryonic membranes surround the it as it undergoes the process of 'turning'. A) presomite headfold stage, 7.5-8.0 days, B) 8.5 day old embryo, C) approximately 8.75 day embryo, D) approximately 9.0 day embryo, E) 9.5 day embryo, F) embryonic layers, extraembryonic tissues and cavities encountered in the embryo illustrated in E).

1. Embryonic endoderm.
2. Embryonic ectoderm and mesoderm.
3. Amniotic cavity.
4. Amnion.
5. Exocoelomic cavity.
6. Yolk sac.
7. Yolk cavity.
8. Ectoplacental cone and trophectoderm derivatives.
9. Reichert's membrane.
10. Parietal (extraembryonic) endoderm.
11. Visceral (extraembryonic) endoderm.
12. Extraembryonic mesodermal component of yolk sac.
13. Mesodermal component of amnion.
14. Ectodermal component of amnion.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1.2 FACTORS AFFECTING PREGNANCY FAILURE

Pregnancy loss occurs in all mammalian species, occuring at particularly high frequencies in humans, with the failure of at least 75% of all conceptions (Bocklage, 1990). Most of these are clinically unrecognized and occur before or at the onset of the next menses. 15-20% of the total are spontaneous abortions (miscarriages) or ectopic pregnancies. In mice, the incidence of postimplantation resorption is approximately 10% (Leonard *et al*, 1971) but this can vary considerably between strains. Preimplantation losses, investigated by counting the number of corpora lutea and comparing with number of implantation sites, can be even higher than postimplantation loss (Leonard *et al*, 1971). Although the timing and extent of prenatal death is much lower in mice many of the factors that lead to death are similar to those in humans. It is beneficial to study the causes of embryonic loss in mice in an attempt to understand loss in humans

Three main routes to pregnancy loss will be discussed, a) the maternal environment and how it can affect pregnancy failure, b) the inappropriate relationship between mother and fetus and c) how an abnormal embryo can affect pregnancy out come.

MATERNAL ENVIRONMENT

The maternal environment may be unable to support the normal development of an embryo. This may be due to an abnormality of the reproductive tract or a hormonal imbalance (Wilmut *et al*, 1986).

Maternal environment has been shown to influence embryonic survival in mice that have been selected for reproductive performance. The response to selection was due to an increase in prenatal mortality rate rather than a reduced ovulation rate. When CN embryos (selected for low litter size) and E embryos (selected for high embryonic survival) were transferred to CN recipient mothers, the number of

live born young was observed when CN embryos were transferred to E recipient mothers, and E embryos showed a reduction in the number of embryos developing to term when transferred to CN recipient mothers (Moler *et al*, 1981). An increase in survival of fetuses from the small litter size line was also evident after daily injections of progesterone during early postimplantation (Michaels *et al*, 1975) providing evidence that normal levels of maternal hormones are also necessary for survival to term.

Reduction in prenatal survival could also be due to the reduced ability of the ageing uterus to carry a fetus to term. An increase in the number of abnormal embryos recovered from ageing female mice has been observed (Tarbert & Krohn, 1966) and this is likely to be a cause of smaller litter sizes observed in older females. However, when apparently normal embryos from older females were transferred to young recipients, the number of live born was not significantly different from the the number of live born from transfer of embryos collected from young donors to young recipients (Gosden, 1979). When embryos from young donors were transferred to older recipients, there was a reduction in live born, suggesting that the capacity of carrying embryos to term in these older females is reduced.

Malformation of the uterus, such as septate or bicornuate anomalies are associated with pregnancy loss in humans, but surgical correction of these can be carried out.

INAPPROPRIATE RELATIONSHIP BETWEEN EMBRYO AND MOTHER

Even if both mother and embryo are normal, loss can still occur.

Overcrowding of the uterus can lead to loss of excess embryos. Embryos that are either too advanced or retarded for the uterine environment may not develop to term. McLaren and Michie (1956) demonstrated that transfer of 3.5 day blastocysts to 2.5

day pseudopregnant uteri gave the best development to term. Timing of development of the embryo to the blastocyst stage is important, if the embryo develops too slowly it may miss the short period of time that the uterus is receptive to implantation.

Immunological factors can also influence prenatal survival. The fetus contains antigens (from the father) which are foreign to the mother, and yet in the majority of pregnancies rejection of the fetus does not occur. However, when *Mus caroli* embryos are transferred to *Mus musculus* uteri, all of the embryos resorb by approximately 10.5d.p.c. Maternal killer T cells have been isolated from the resorptions and a deficiency of suppressor non-T cells in the decidua were reported (Croy *et al* , 1982, 1985, Clark, *et al*, 1986), although the actual mechanisms involved in the resorption of the *Mus caroli* embryos is still unclear.

In the CBA/J female x DBA/2 male mouse model of embryonic loss, a high incidence of resorptions occur (~30%) but not complete resorption of the litter (Clark et al, 1980). The majority of resorptions occur within a time window where there is a timing mismatch between the appearance of a novel transforming growth factor-β (TGF- β) at day 10.5 and tumour necrosis factor-α (TNF-α) at day 8.5. In CBA/J x C3H/HeJ matings these two factors arise simultaneously (Clark *et al*, 1991) and a high resorption rate is not observed. It has been suggested that in the absence of the novel TGF-β, TNF–α activates Natural Killer cells to become lymphokine activated killer cells (LAKS) which can then destroy the trophoblast. In this model it is possible to protect against resorption by immunizing with spleen cells from BALB/c, BALB/c x DBA/2, and BALB/c x F1 mice (Chaouat *et al*, 1985, 1989, Kiger *et al*, 1985).

Another example of immunological response resulting in death of the fetus is observed in immunodeficient SCID mice on a CB-17 background. This

unexpectedly high rate of resorption could not be prevented by treatment with factors that are effective in reducing resorption in the CBA/J x DBA/2 system, but were reduced by outcrossing mice with a different (C57BL/6) background. However, TNFα is also released prior to TGF-β in these mice (Clark *et al*, 1992).

EMBRYONIC ABNORMALITIES

Pregnancy may fail because an embryo is abnormal. Abnormalities may arise due to either chromosomal anomalies or because of specific mutations. These could have arisen from a change in the nucleus of either gamete or a spontaneous mutation occurring within the fertilized ova.

Chromosomal anomalies can be due to either loss (monosomy) or addition (trisomy) of a single chromosome, or loss (haploidy) or addition (polyploidy) of a whole set of chromosomes.

Very few trisomic and monosomic embryos reach term, although normal development to the blastocyst stage can occur. Autosomal monosomics die at around the time of implantation (but XO mice are live born), whereas most trisomics die between about 10 days and birth (Gropp, 1976, Fundele *et al*, 1985, see review by Dyban & Baranov, 1987). When missing a full set of chromosomes (haploidy), development past the blastocyst stage is rare. Triploid embryos can develop beyond implantation but die between 10 and 12 d.p.c. (Wroblewska, 1971; Kaufman & Speirs, 1987 and Kaufman, 1991), although recovery of a 14 day old triploid mouse embryo has been reported (Bos-Mikich & Whittingham, 1992). Most tetraploid embryos appear to die early in development with abnormalities becoming apparent at approximately 8.5 d.p.c. (Tarkowski *et al*, 1977 and James *et al,* 1992), and these rarely develop past mid-gestation (Kaufman & Webb, 1990). There has however been one report of tetraploid embryos which have survived beyond implantation and

to term (Snow, 1975) with abnormalities becoming apparent at approximately 13.5 days.

Trisomy contributes to about one third of spontaneously aborted fetuses in humans. These rarely survive beyond 22 weeks (Loidero *et al*, 1989) and are aborted as severely retarded but well formed fetuses. Trisomy 21, however, can survive to birth but results in physical and mental retardation.

A number of specific mutations are known which disrupt development of the embryo, the oldest of which arose spontaneously. Mutations can be produced via different types of mutagenesis for example induced by chemicals, radiation, or more recently by transgenic technology. Mutations that result in embryonic death have been documented from almost every stage of development except those occuring just prior to birth (between days 16 and 18) (see reviews by McLaren, 1976a, Magnuson & Epstein, 1981, Magnuson, 1986, and Copp, 1995).

Mutations causing death during early preimplantation development are thought to disrupt basic cellular functions. Embryos homozygous for oligosyndactyly (*Os*) appear normal until the blastocyst stage. Cells then become pycnotic and lack a nuclear membrane and nucleolus. The mutation causes mitotic arrest and the primary effect is thought to be on the mitotic spindle (Magnuson & Epstein, 1984). Embryos heterozygous for *Os* have abnormalities in limb development (Gruneberg, 1961).

The defects caused by many early acting mutations are both cell autonomous and cell lethal for example lethal yellow (A^y) embryos. A^y/A^y embryos die shortly after implantation. In chimaera experiments A^y/A^y inner cell masses were able to survive when combined with normal trophectoderm (Papaioannou & Gardner, 1976), suggesting that death of A^y/A^y embryos is linked to a trophectoderm deficiency. However, when chimaeras were produced by aggregating normal and homozygous

A^y/A^y embryos together, no homozygous A^y/A^y cells were observed at 9.5days or term (Barsh *et al*, 1990).

Later acting mutations can be classified into four catagories (Copp, 1995), (1) those causing death during the peri-implantation period resulting in the embryo dying shortly after implantation because a proper connection does not form between the trophoblast and decidua and therefore nutrition becomes a problem; (2) failure at gastrulation, caused by defects in primitive streak formation, incomplete mesoderm formation and failure of mesoderm cells to be displaced away from the primitive streak leading to the absence of an embryonic blood circulation; (3) failure to establish the chorioallantoic placenta and (4) failure of cardiovascular circulation or liver haematopoiesis.

An example of peri-implantation failure is the c^{6H} mutation at the albino locus on chromosome 7. Homozygous embryos are first detected at 6.5 - 7.0 d.p.c. because of abnormalities of the ectoplacental cone and the parietal endoderm. The ectoplacental cone was either reduced in size or completely absent in these embryos (Lewis *et al*, 1976). Homozygous lethal yellow embryos also die during the peri-implantation period. In both of these cases, development to the blastocyst stage occurs, but connection between the embryo and mother is impaired and results in death of the embryos.

The strain 413-d insertional mutation results in abnormal embryos at 7.0 d.p.c. which fail to form mesoderm, although both embryonic ectoderm and endoderm over proliferate (Iannaconne *et al,* 1992). The 413d mutant embryos also fail to show morphological evidence of a primitive streak (Conlon *et al*, 1994). The gene *nodal*, a novel member of the TGF-β growth family (Zhou *et al*, 1993) has been found to map closely to the 413-d retroviral insertion. Disruption of nodal is likely to be involved in the abnormal 413d phenotype.

Mouse embryos lacking *msd* (mesoderm deficiency) fail to produce mesoderm but form well defined extraembryonic and thickened embryonic ectoderm. These embryos show signs of abnormalities at 7.5 - 8.0 d.p.c. and are completely resorbed by approximately 11.0 d.p.c. (Holdener *et al*,1994).

The embryonic ectoderm (*eed*) deletion mutant (Niswander *et al*, 1988) has abnormalities in the primitve streak and results in incomplete mesoderm formation. At 7.5d.p.c , the abnormal phenotype is not always apparent, but becomes more obvious by 8.5d.p.c. when the embryos can be identified by having a small embryonic ectoderm and form only small amounts of mesoderm. The extraembryonic structures are more developed with the formation of the amnion, allantois and chorion. The deletion maps to the albino deletion complex on chromosome 7. Correct expression of a proximal primitive streak marker (*Flk1*) was observed, but expression of distal primitive streak markers (*goosecoid* and fibroblast growth factor 4 (*Fgf4*)) were not expressed (Faust *et al*, 1995). Only sparse embryonic mesoderm was observed but was found to be correctly specified suggesting a role for the deleted gene product in primitive streak formation and/or function (Faust *et al*, 1995).

Mutants homozygous for Brachyury (*T*) die at about 10 d.p.c. and lack a notochord and structures that form from the posterior part of the primitive streak. The primitive streak becomes abnormally thickened, and the allantois fails to join with the ectoplacental cone. However, *T* expression is observed from the onset of gastrulation in the area of the primitive streak and in the axial mesoderm. Results from injection chimaera studies suggest a role for Brachyury in the normal morphogenetic movements of mesodermal cells during gastrulation (Wilson *et al*, 1995). Death of the mutant embryos is thought to be caused by the inability to form the chorioallantoic placenta.

Death during the later stages of development is associated with a variety of defects, including abnormalities of the central nervous system, lungs, gut, limbs and urogenital system, but these defects are not thought to be the underlying cause of death as other mutants can survive with defects in these organ systems. Failure of the cardiovascular system, or liver haematopoiesis, or both appear to be the underlying cause of death (Copp, 1995).

1.3 ENERGY METABOLISM OF EMBRYOS

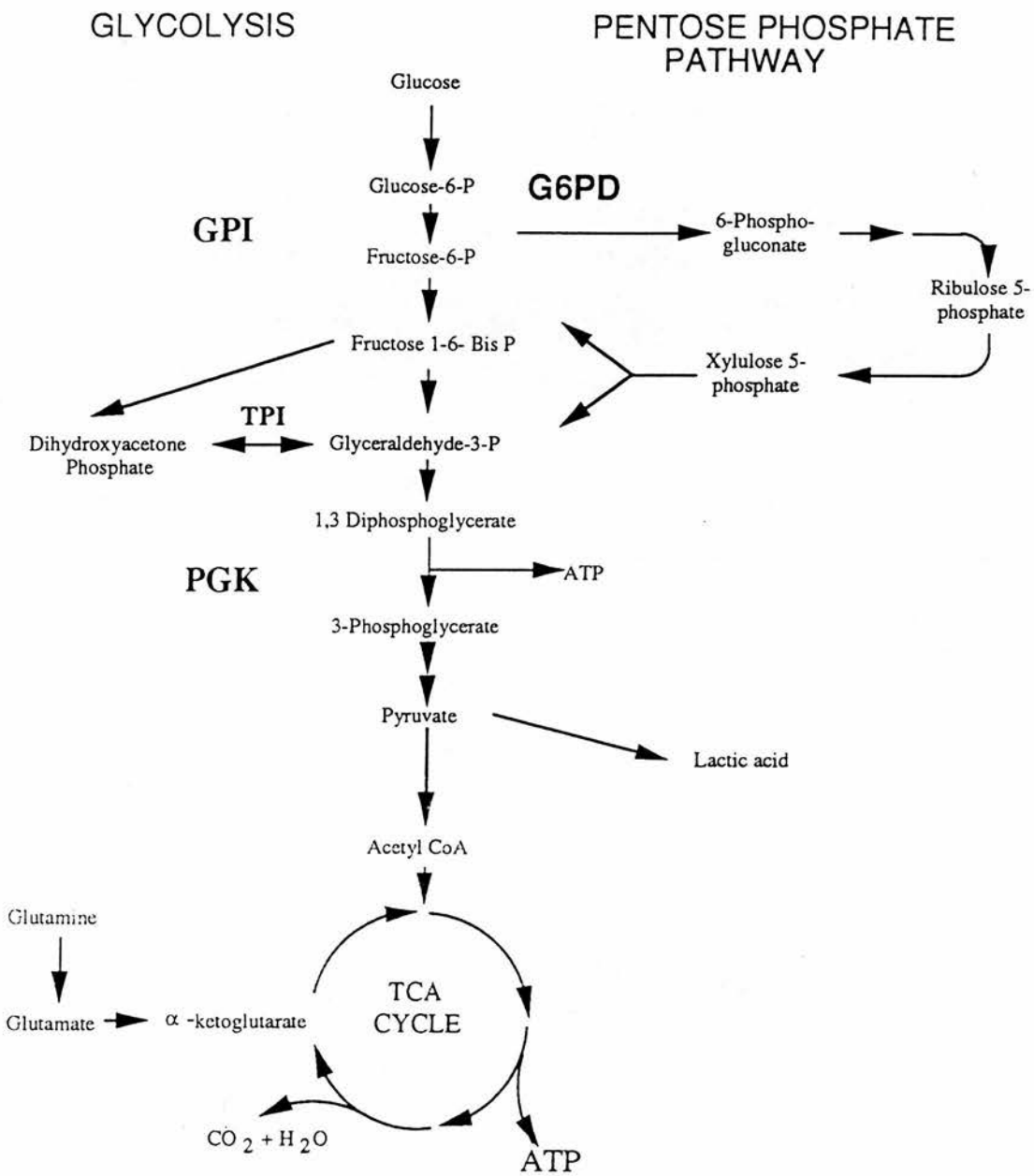
In most animal cells, energy is produced by glycolytic breakdown to form pyruvate, which under aerobic conditions, is oxidised via the mitochondrial tricarboxylic acid (TCA) cycle (Fig 1.3). Energy is produced both by glycolysis and the TCA cycle under these conditions, but more energy is produced by the TCA cycle.

During development of the embryo, the energy requirements of the embryo change. Knowledge of these requirements has come from *in vitro* experiments, directly using embryos collected from the genital tract or from culturing embryos for a few days.

Fertilized and unfertilized oocytes use pyruvate predominantly as the major energy source (Biggers *et al*, 1967), but if the oocyte is surrounded by follicle cells, glucose can be utilized (Donahue & Stern, 1968). This illustrates that the follicle cells can communicate with the oocyte by allowing the passage of substrates. The normal mouse embryo is unable to use glucose as the sole energy source until the 8-cell stage (Brinster & Thomson, 1966) and therefore energy production at these stages depends on the tricarboxylic acid (TCA) cycle, which functions throughout preimplantation development. This has been demonstrated by experiments measuring the uptake of various substrates by groups of embryos. Two cell up to 8-cell embryos also have a preference for pyruvate, although they can also utilize lactate and glucose to a lesser extent (Brinster *et al*, 1967).

Fig 1.3. Simplified diagrammatic representation of glycoysis and TCA cycle.

-P = Phosphate
G6PD = Glucose-6 phosphate dehydrogenase
GPI = Glucose phosphate isomerase
PGK = Phosphoglycerate kinase
TCA = Tricarboxylic acid
TPI = Triose phosphate isomerase


GLYCOLYSIS
PENTOSE PHOSPHATE PATHWAY
Glucose
Glucose-6-P
G6PD
GPI
6-Phospho-gluconate
Fructose-6-P
Ribulose 5-phosphate
Fructose 1-6- Bis P
Xylulose 5-phosphate
TPI
Dihydroxyacetone Phosphate
Glyceraldehyde-3-P
1,3 Diphosphoglycerate
PGK
ATP
3-Phosphoglycerate
Pyruvate
Lactic acid
Acetyl CoA
Glutamine
Glutamate
α -ketoglutarate
TCA CYCLE
CO 2 + H 2O
ATP

From the blastocyst stage, pyruvate (or lactate) can no longer support development of the embryo alone, and an increase in glucose utilization occurs. The formation of lactate also increases at this time (Biggers & Stern, 1973), which indicates a switch from aerobic to anaerobic metabolism. The switch from pyruvate to glucose metabolism occurs at around the time of compaction (Leese & Barton, 1984). The energy metabolism of early post-implantation embryos (6.5 - 9.5 days) is dependant on glycolysis as shown by CO_2 and lactate production (Clough & Whittingham, 1983). The increase in anaerobic glycolysis at this time is assumed to be in response to the anoxic conditions the embryo encounters during the early implantation stage (Merkle & Pretsch, 1992) before the formation of the chorio-allantoic placenta (Cox & Guneberg, 1972, Ellington, 1987). Once the placenta is functioning, conditions would once again be aerobic and the TCA cycle would again become active.

Several glycolytic deficient mutants have been described in the mouse. Charles and Pretsch (1987) produced four Triosephosphate Isomerase (TPI) mutants in *Mus musculus*. Mice heterozygous for the TPI mutation had 50% of wild type activity of the enzyme. Breeding data and genetic analysis of offspring from intercrossing 2 heterozygotes proved that homozygotes were embryo lethal, as none were found at term. It is thought that death of these mutants occurs at an early post-implantation stage of development but this was not tested directly. TPI occurs later in the glycolytic pathway than GPI and catalyses the interconversion of dihydroxy-acetone phosphate (DHAP) to glyceraldehyde-3-phosphate (GAP) (Fig 1.2). Death of the cells and embryo may be due to accumulation of DHAP.

Glucose-6-phosphate dehydrogenase (G6PD) deficient mutants have also been described. One mutant of interest that may be a candidate model for human G6PD, is the X-linked G6PD mutant, which in the hemizygous, heterozygous and

homozygous states has 15, 60 and 15% G6PD activity respectively (Pretsch *et al*, 1988).

1.4 GLUCOSE PHOSPHATE ISOMERASE

Glucose phosphate isomerase (GPI) (E.C. No. 5.3.1.9., phosophoglucose isomerase) is an important glycolytic enzyme. It catalyzes the interconversion of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), and therefore it's activity coupled with other factors, determines the amount of G6P that enters the glycolytic pathway (Fig 1.2). It is a dimeric enzyme composed of identical subunits with a molecular weight of about 134kd. The gene encoding GPI is situated on chromosome 19 in man (McMorris *et al*, 1973) and chromosome 7 in the mouse (De Lorenzo & Ruddle, 1969, Hutton & Roderick, 1970). It is a housekeeping gene and important in all cells involved in glycolysis and gluconeogenesis.

The locus controlling GPI in the mouse is designated *Gpi -1s* and several different alleles have been described including, $Gpi\text{-}1s^a$, $Gpi\text{-}1s^b$ and $Gpi\text{-}1s^c$ (Carter & Parr, 1967; De Lorenzo & Ruddle, 1969; Padua et al, 1978). Dimers can be separated electrophoretically (Chapman, Whitten & Ruddle, 1971) because they differ in their electrophoretic mobility. Under conditions where GPI migrates to the cathode, migration occurs in the order GPI-1C (fastest), GPI-1B and GPI-1A (slowest). The relative stability of GPI-1 dimers was found to be AA>AB>BB>AC≥BC>CC (West & Flockhart, 1989). Antibody techniques have been used to try to identify the genotype of cells in chimaeras and to look at the expression of GPI in different types of cells, but these gave mixed results. Gearhart *et al.* (1981) developed antisera specific for the GPI-1B variant but not for GPI-1A and Conrad *et al.* (1987) found high serological similarities among the three allozymes. GPI-1B and GPI-1C could be differentiated from GPI-1A, and GPI-1A and GPI-1B could be differentiated from 1C, but GPI-1A and GPI-1B react identically with rabbit anti-GPI-1B antisera.

The onset of expression of embryonic genes was investigated by observing when protein coded by the paternal allele was first detectable. Chapman *et al*, (1971) detected *Gpi-1s* from the paternal locus at day 5 in the late blastocyst (in culture) and suggested that transcription must be occuring prior to this at about day 4. However, as the techniques for identifying the GPI allozymes became more sensitive, paternal *Gpi-1s* was identified at earlier stages, Brinster (1973) detected paternally coded enzyme at the 8-cell stage providing evidence that the paternal genome is activated earlier than had been previously thought. The oocyte-coded GPI still remains when the embryonic GPI is detectable and is not exhausted until ~5.5-6.5d.p.c. (West and Green 1983; Duboule and Burki, 1985; Gilbert and Solter, 1985; West et al 1986; West and Flockhart,1989).

Deficiency of GPI in humans results in a congenital disorder with the typical manifestations of non-spherolytic anaemia of varying degrees of severity, first described by Baugham *et al*, (1967). It is characterized by diminished erythrocyte number, lower haemoglobin, a higher number of reticulocytes and plasma bilirubin concentrations.

Several different mutations of *Gpi-1s* have been induced using triethylenemelamine (TEM) (Soares, 1979, Merkle & Pretsch, 1992) and ethylnitrosourea (ENU) to try to produce an animal model of this disease and also to try to determine how an individual would be affected by a mutation in this gene. TEM is a potent mutagen in mice and has been shown to cause chromosomal and specific locus mutations in mice (Cattanach, 1967), and ENU is also an effective inducer of specific locus mutations in mice (Russell *et al.*, 1989).

In mutation experiments, (Peters *et al*, 1986) male mice of inbred strains 101/H and C3H/HeH were injected intraperitoneally with 200 - 250mg of ENU. Two months later these males were mated and offspring were analysed by electrophoresis of a

blood sample. Thirteen mutations were found, 5 of these were at the *Gpi-1s* locus and resulted in complete loss of protein or enzyme activity. One of these mutants, *Gpi-1s* ^{am1H}, produced viable and fertile heterozygous offspring, but no homozygotes were produced (Peters & Ball, 1986).

Merkle and Pretsch, (1992) also produced *Gpi-1s* mutants by treating males with triethylenemelamine and a single dose of 6Gy gamma rays 24 hr later.

Studies investigating the development of embryos carrying these null alleles found that they died shortly after implantation (West *et al.*1990; Merkle & Pretsch, 1992) and formed an empty sac structure. Although the null embryos cannot produce their own GPI, because they lack the appropriate gene, they survived until 7.5-8.5 d.p.c.(West *et al.* 1990), beyond the time when oocyte coded GPI is depleted. Some cells appeared to survive for longer until 10.5 d.p.c.

During early post-implantation development, the embryo is dependant on anaerobic glycolysis (Clough & Whittingham, 1983). These mutant embryos however have a block in the glycolytic pathway because they cannot produce their own GPI (Peters *et al*,1986, Merkle & Pretsch, 1992). The pentose phosphate pathway has been shown to be active during implantation (Clough & Whittingham, 1983) but may only generate low levels of substrate for glycolysis, as its main function is to generate reducing power in extramitochondrial cytoplasm in the form of NADPH (Lehninger, 1970). Very little energy, if any, will be produced via the TCA cycle because of the anaerobic conditions, and if no other substrates are available to the embryos death will occur due to a lack of energy. Conditions will be anaerobic until formation of the placenta (Cox & Guneberg, 1972, Ellington, 1987). Survival of some structures is apparent however (West, 1993). These tissues may survive better because they are close to the maternal tissues and so can receive nutrients and oxygen from the mother and are therefore able to utilize the TCA cycle. Alternatively, their energy

requirement may be less than other tissues within the embryo leading them to survive slightly longer. Further study to attempt to characterize whether the tissues on the outside of the mutant embryo utilize different energy pathways will be described in Chapter 3.

It has been shown that cultured animal cells that are deficient in GPI can use alternative metabolic pathways. Pousseygur *et al* (1980) looking at mutant chinese hamster fibroblasts that were deficient in GPI found that the mutant cells were able to utilize glucose at a very low level using the pentose phosphate shunt. Chinese hamster ovary cells (CHO) that lack both GPI and PGK (Fig 1.3) can survive when cultured in glucose but this seems to be due to the presence of other substrates in the culture medium (eg. glutamine) which the cells can then utilize via the TCA cycle (Morgan *et al,* 1981).

Bacterial strains that have a GPI deficiency have also been described. A mutant strain of *E. coli* has been reported that that has no GPI activity. It grows at one third the rate of the normal strain mainly using the pentose phosphate shunt to metabolize carbohydrate (Fraenkel & Levisohn 1967). The requirement of GPI in these organisms does not lead to death of the cells but a reduced growth rate. *Saccharomyces cerevisiae* mutants that lack GPI grow on fructose if provided with a small amount of glucose. Large amounts of glucose led to the accumulation of G6P and inhibition of growth (Herrera and Pascual, 1978).

1.5 EXPERIMENTAL CHIMAERAS

Chimaeras were used in this thesis to test the developmental potential of cells homozygous for a null allele for *Gpi-1s*. Aggregation chimaeras were produced between normal and and genetically abnormal embryos to determine whether, being homozygous for the null allele is cell lethal as well as embryo lethal.

The term 'chimaera' means composite animal, where an individual has cell populations derived from more than one fertilized egg. There are two different types of chimaeras, 'primary chimaerism' where two or more embryos are combined at a very early stage (eg. at the 4- or 8-cell stage), and 'secondary chimaerism' where fetal or adult tissues are combined that have undergone a period of organogenesis. Mosaics differ from chimaeras in that they arise from a single embryo in which a developmental change has occured causing a mosaic phenotype. This could include somatic mutation or may arise by the spontaneous inactivation of an X chromosome in female conceptuses.

The first experimental mouse chimaeras were produced in 1961 by Tarkowski. Cleaving embryos with their zona pellucidae removed were aggregated and cultured to the blastocyst stage. These were then transfered to pseudopregnant recipients (females that had been mated to vasectomized males) and at term chimaerism was detected by presence of pigment in retinal pigmented epithelium. Various changes were made to this initial technique, including using pronase to remove the zona pellucida rather than the mechanical removal of this membrane, to make the technique easier and to increase the chances of embryo survival (Mintz, 1962).

Chimaeras can also be produced by the injection of groups or individual cells into the the blastocoel cavity. This was first demonstrated by Gardner (1968). The injected cells however only give rise to tissues derived from inner cell mass. Embryonic stem cells (ES cells), which are pluripotent (Evans & Kaufman, 1981, Martin, 1981) can also be used to form chimaeras. They can be either injected into the blastocoel of a mouse embryo to form an injection chimaera (Bradley *et al*, 1984), or the cells can be sandwiched between two 4- or 8-cell embryos to form aggregation chimaeras (Stewart, 1980, Fujii & Martin, 1980 using embryonal carcinoma (EC) cells, Spindle, 1982)

Chimaeras are useful developmental tools when the two cell populations used can be distinguished from one another. By studying the developmental fate of the two cell populations, the origin of tissues and cell lineages can be traced. The developmental potential of embryonic cells with different genotypes can be studied in chimaeric individuals at various stages of gestation. Aggregation chimaeras have the advantage over injection chimaeras, that cells can contribute to all lineages and are not restricted to the inner cell mass derived tissues.

By aggregating embryos, one homozygous for a mutant allele and the other wild type, it is possible to investigate whether mutant cells can be rescued by the presence of normal cells or whether only development of the wild type cells is observed. Various types of chimaeras have been produced by aggregating an abnormal embryo with a normal embryo and insights gained into the potential of the abnormal cells. For example, when embryos from A^y/a x A^y/a were aggregated to embryos from *A/A* x *A/A* matings, no $A^y/A^y \leftrightarrow A/A$ chimaeras were identified at term, demonstrating that homozygous A^y/A^y cells cannot be rescued in a chimaeric environment (Barsh *et al*, 1990).

When parthenogenetic $\leftrightarrow$ normal chimaeras are produced, at 12.5d.p.c. the parthenogenetic cells were observed to contribute to the embryo and yolk sac mesoderm, suggesting that selection against parthenogenetic cells to the primitive endoderm lineage occurs (Surani *et al*, 1988, Thomson & Solter, 1988a, Fundele *et al*, 1990). At term and into adulthood, the contribution of parthenogenetic cells in tissues that undergo self renewal declines (Fundele *et al*, 1989). In androgenetic $\leftrightarrow$ normal chimaeras, androgenetic cells were found in trophoblast and yolk sac and only one case were any androgenetic cells found in the fetus (Surani *et al*, 1988, Thomson & Solter, 1989). It appears that parthenogenetic and androgenetic development complement each other, and if the two populations of cells could in some way be joined, then a normal fetus would be formed.

Chimaeras, however can only be studied, if the two populations of cells can be distinguished from each other, that is if one of the embryos is marked. Several criteria must be fulfilled before a candidate marker can be considered (McLaren, 1976b, West, 1984). All cell and tissue descendants from the original labelled cell should carry the marker. It should be stably transmitted to daughter cells and not degrade upon subsequent divisions It should be cell autonomous, cell localized and not leak into surrounding cells, and the presence of the marker should be developmentally neutral having no affect, detrimental or otherwise on the development of the chimaera in any way. The marker should also be easy to detect.

Many different markers have been used with varying degrees of success in previous studies. Vital dyes, tritiated thymidine and horseradish peroxidase (HRP) have been used. The problem with these cell markers are that they are diluted out in subsequent divisions so that only short culture periods can be analysed when cells are marked in this way. For example, in the case of HRP up to 64 - fold dilution can be detected reliably (Lawson 1986) after this it's reliability reduces.There is also the added problem of cellular degradation leading to loss of the marker.

Genetic markers and biochemical markers have also been used. Pigment fulfils most of the requirements necessary for a marker but is restricted to the few tissues that are actually pigmented, for example the retinal epithelium, the inner ear and in the coat. Pigment in the retinal epithelium is a good marker as it remains localized within cells. It can be visualized as early as 12.5d.p.c. where it forms a single layer. It can also provide spatial information about its distribution. In the coat two types of melanocytes may colonize a single hair giving rise to uneven pigmentation (McLaren, 1976) making this a less precise marker. Also, it can only be used once the chimaeras have reached term.

Many biochemical cell markers have been used to indirectly assay the proportion of different cell types in chimaeras, such as malic enzyme (Peterson,1974, Gardner, 1984). One that is widely used is glucose phosphate isomerase (GPI). It is a dimer and as already mentioned several different monomers have been identified including, GPI-1A, GPI-1B and GPI-1C (Carter & Parr, 1967; De Lorenzo & Ruddle, 1969; Padua *et al*, 1978). Different homodimers and heterodimers can be separated by electrophoresis and visualized as stained bands (allozymes) of GPI activity (Chapman, Whitten & Ruddle, 1971). Current electrophoresis techniques are sufficiently sensitive to detect a minor allozyme that contributes as little as 3% of the total activity in typical tissue homogenates. The disadvantage with using electrophoretic markers, is that no spatial information can be obtained because the tissues are homogenized before assaying. Contamination of the tissue types can occur if the dissection of parts is not entirely accurate and a false result gained. An average value is obtained from all the tissues present in the sample which may not reflect the exact proportions of the cell populations within the sample.

The first DNA-based *in situ* cell marker system was described by Rossant *et al* (1983). DNA satellite probes were used to identify the different cell types in *Mus musculus* $\leftrightarrow$ *Mus caroli* chimaeras.This system had the advantage over other markers because it can be detected in histological sections and the spatial distribution of the marker can be analysed. It also fulfilled many of the criteria required for a cell marker, it is cell autonomous and is confined to the cell nucleus, it is not diluted out in subsequent divisions and most importantly it can be detected in all tissues. It is not clear however, whether the amount of cell mixing in interspecific and intraspecific chimaeras is comparable.

One 'marked' transgenic mouse strain that is widely used is strain 83 produced by Lo (1986). Incorporated into one site on chromosome 3 are 1000 copies of the mouse β - major globin gene (TgN(Hbb-b1)83Clo). This transgene again fulfils most of the

criteria previously described (Thomson & Solter, 1988b). A problem with inserting large amounts of exogenous DNA into chromosomes is that it is unclear whether it will affect the development of the embryo or whether it will remain developmentally neutral. However a recent study by West and Keighren (unpublished results) have shown that mice heterozygous for the transgene are not adversely affected by its presence. The efficiency of scoring the number of positive nuclei present from a histological section will depend on the thickness of the section, not all of the sections will have the part of the nucleus that contains the transgene and therefore there will be some false negatives scored. But this can be accounted for by always including a positive control slide to estimate the proportion of transgenic signal that is present.

Other markers that are becoming more widely used that will not have the problems of a nuclear marker are transgenes that express *E. coli Lac Z* gene (e.g. Beddington *et al* 1989, Gossler *et al*, 1989, Freidrich and Soriano, 1991). When lac Z is expressed in the cytoplasm under the control of an ubiquitous promoter, it provides an excellent lineage marker for use in chimaera studies, as long as its expression is not down regulated in a tissue specific or age specific pattern. Staining with X - Gal gives rise to a blue colour. Such markers are not without problems and, for example, down regulation of expression may occur in some tissues.

Several cell markers were used in this study and these were chosen because of their suitability for our needs. Chimaeras were produced and analysed at 12.5 d.p.c. and at term. Eye pigmentation was used to estimate what proportion of the cells in the chimaeric embryos were derived from the pigmented population (Tarkowski, 1964). Estimate of the % eye pigment in a single section from 12.5 day chimaeric embryos has been shown to agree well with the corresponding % GPI in the fetal trunk (Hodson, Keighren & West, unpublished). Pigment is easy to detect and does not need any enzymatic reactions for visualization. Pigment granules are dark in colour and can be seen easily in sections stained with haematoxylin and eosin. Initially an

estimate of the pigment was made at the time of dissection, then another estimate was made from a histological section. Glucose phosphate isomerase was also used as a marker. The chimaeras being investigated were produced from strains containing different electrophoretic variants of this enzyme. The proportions of cells that are derived from each embryo can therefore be estimated and also the genotype of the embryo. The pigmented component of the chimaera also carried the b major globin marker (Lo,1986) which can be visualized by DNA *in situ* hybridisation. In homozygous GPI null $\leftrightarrow$ normal chimaeras, only the proportion of cells derived from the normal component of the chimaera will be identified by GPI electrophoresis, the presence of pigment and detection transgene were used to identify these chimaeras.

EXPERIMENTAL AIMS

The experimental aims of this thesis were (1) to characterize the abnormalities apparent in mutant homozygous GPI null embryos and (2) to produce aggregation chimaeras between the mutant and normal embryos to determine whether homozygous null cells can be rescued in the presence of normal cells.

The experimental work described in this thesis is divided into two parts. Part I (Chapters 2-4) deals with issues that relate to the developmental potential of homozygous GPI null **embryos**. Part II (Chapters 5-10) deals with the developmental potential of homozygous GPI null **cells** in aggregation chimaeras.

PART I; Chapter 2 describes the histological characterization of the mutant homozygous GPI null embryos. In Chapter 3 an attempt to characterize the metabolic activity in the GPI null mutant and their extraembryonic membranes was made. However, no conclusive information was gained from this study. Another embryonic lethal was examined in Chapter 4, the CBA/J x DBA/2 immunological model of embryo loss. The histology of the abnormal embryos produced in this cross were compared to the mutant homozygous GPI null embryos. An unsuccessful attempt was made to determine whether TNF-α expression in the mutant homozygous GPI null embryos was similar to that of the CBA/J x DBA/2 resorbing embryos.

PART II; Chapters 5 and 6 identify probable homozygous null chimaeras at 12.5 d.p.c., using a variety of techniques, GPI analysis, eye pigmentation and *in situ* hybridisation analysis of a transgenic marker. Chapters 7, 8, and 9 describe the analysis of the normal (+/+), heterozygous null (+/*m*) and homozygous null (*m*/*m*) fetal chimaeras. Adult chimaeras were produced and one homozygous null female was identified and described in Chapter 10.

PART I

THE DEVELOPMENTAL POTENTIAL OF HOMOZYGOUS GPI NULL EMBRYOS

CHAPTER 2

THE HISTOLOGICAL CHARACTERIZATION OF EMBRYOS HOMOZYGOUS FOR A NULL ALLELE OF GLUCOSE PHOSPHATE ISOMERASE

2.1 INTRODUCTION

Glucose phosphate isomerase (GPI) is an important glycolytic enzyme which catalyzes the reversible interconversion of glucose-6-phosphate (G6P) to fructose-6-phosphate (F6P) and therefore its activity, coupled with other factors, determines the amount of G6P that enters the glycolytic pathway (Fig1.3).

In mouse embryos, the embryonic gene (*Gpi - 1s*), encoding GPI is activated between 2.5 and 3.5 days *post coitum* (d.*p.c.*). Before this time, the enzyme is entirely oocyte-coded and some oocyte coded GPI remains until approximately 5.5 - 6.5 d.p.c.(Chapman, Whitten and Ruddle, 1971; Brinster, 1973; West and Green, 1983; Duboule and Burki, 1985; Gilbert and Solter, 1985; West, Leask and Green, 1986; West and Flockhart, 1989).

A null mutation in the *Gpi - 1s* gene arose from mutagenicity experiments using E.N.U (Ethylnitrosourea) and resulted in complete loss of gene product. This null allele was designated *Gpi - 1s* $^{a\text{-}m1H}$. Offspring heterozygous for the mutation were fully viable and fertile, but homozygotes were unknown at term (Peters and Ball, 1986). Previous studies, employing biochemical techniques and relying on gross morphological observations, have shown that the homozygous embryos die shortly after implantation (West *et al* 1990; Merkle and Pretsch, 1992) due to lack of GPI. It was found that no residual GPI activity remained after 6.5d.p.c. in embryos

homozygous for the null allele (West *et al* 1990). Certain extraembryonic tissues survived until 10.5 d.p.c. but by 11.5 and 12.5 d.p.c. no structures remained (West, 1993).

This study was undertaken to identify the first histological signs of developmental abnormalities in the homozygous mutant embryos, in order to try to determine when an intact glycolytic pathway was required.

2.2 MATERIALS AND METHODS

MICE

The mutant null allele, *Gpi-1s*$^{a\text{-}m1H}$ (abbreviated to *'m'* in this paper) arose from the *Gpi-1s*^{a} allele, as reported by Peters and Ball (1986). The stock that carries the null mutation was produced by crossing a *Gpi-1s*^{a}/*Gpi-1s*$^{a\text{-}m1H}$ (*a/m*) heterozygote with an inbred C57BL/OlaWs mouse, homozygous for *Gpi-1s*^{b}. Heterozygous *Gpi-1s*^{b}/*Gpi-1s*$^{a\text{-}m1H}$ (*b/m*) offspring were crossed with C57BL/Ola-*Gpi-1s*^{a}/Ws partially congenic (N8) mice, homozygous for *Gpi-1s*^{a} (West *et al.*, 1990), and subsequent generations were crossed alternately to C57BL/OlaWs and C57BL/Ola-*Gpi-1s*^{a}/Ws. The resultant partially congenic C57BL/Ola-*Gpi-1s*$^{a\text{-}m1H}$/Ws mice were heterozygous for the null mutation and either the *Gpi-1s*^{a} or the *Gpi-1s*^{b} allele. They are designated *a/m* and *b/m* respectively.

In the experimental matings, heterozygous *a/m* females were crossed with heterozygous *b/m* males to produce *a/b*, *a/m*, *m/b* and homozygous *m/m* null embryos. In the control matings, heterozygous *a/m* females were crossed with homozygous *b/b* males to produce *a/b* and *m/b* embryos.

EMBRYO COLLECTION

Females were checked daily for the presence of a vaginal plug. The day of vaginal plug detection was designated as 0.5 days *post coitum* (d.p.c.) as mating was assumed to have occurred the preceding night. Pregnant females were killed by cervical dislocation on 6.5, 7.5, 8.5, and 9.5 d.p.c. Embryos within their decidual swelling were dissected free from the uterus in phosphate buffered saline or Hepes buffered M2 medium (Quinn, Barros and Whittingham, 1982).

HISTOLOGY

Embryos and decidual swellings were fixed in 3:1 ethanol:acetic acid overnight. The samples were dehydrated through a graded series of ethanol, followed by HistoClear (National Diagnostic), immersed in 50:50 Histo-Clear:paraffin wax mixture and impregnated with paraffin wax under vacuum. After this processing, the samples were embedded in wax and stored at 4°C. Sections of the tissue were cut at 7μm and stained with haematoxylin and eosin.

Embryos in Experiment 1 (West *et al,* 1990) were classified after dissection by their morphological appearance as normal, small or abnormal; dead embryos were counted as abnormal. Embryos in Experiment 2 were classified by their appearance in histological section as normal or abnormal; retarded and dead embryos were counted as abnormal.

STATISTICAL ANALYSIS

Statistical tests (χ^2 or Fisher's Exact test) were performed on an Apple Macintosh computer using the statistical package "MultiStat" (Biosoft, Cambridge, UK).

The following formula (based on that used by Lyon, 1970 to calculate the number of induced mutations) was used to calculate the % of abnormal embryos induced by the

experimental cross and corrects for the presence of sporadic abnormal embryos in the control cross:

$$\left[1 - \left(\frac{\text{Normal embryos in experimental cross}}{\text{Implantation sites in experimental cross}} \Big/ \frac{\text{Normal embryos in control cross}}{\text{Implantation sites in control cross}}\right)\right] \times 100$$

2.3 RESULTS

To characterize the earliest sign of abnormality, in the development of the homozygous null embryos, which represents the most direct consequence of the mutation, embryos were examined from 6.5 d.p.c. through to 9.5 d.p.c. in control and experimental crosses. The frequencies of abnormal embryos was compared between the two crosses and examined the histology of the abnormal embryos. The frequencies of abnormal conceptuses in the experimental and control crosses are shown in Table 2.1. Data in Experiment 1 are taken from West *et al* (1990) where the *a/m* females were superovulated and mated to either *b/b* or *b/m* males. These embryos were classified morphologically before being analysed biochemically but no histology was undertaken. Embryos in Experiment 2 were from natural matings, without superovulation and were classified by their appearance in histological sections.

At 6.5 d.p.c there was no significant difference in the frequency of abnormal embryos between the control and experimental groups in either Experiment 1 or 2. However, at 7.5 days, both experiments showed a statistically significant increase in the proportion of abnormal embryos in the experimental cross. Also, after correcting for the presence of abnormals in the control groups, the calculated percentage of "induced" abnormals was close to the expected 25% frequency of homozygous *m/m* embryos. In Experiment 1, where embryos were classified separately as normal, small or abnormal, most of the small or abnormal group were classified as small. This suggests that the first morphological effects of the GPI defect may simply be retarded development rather than some gross morphological aberration. In both experiments the frequency of abnormal embryos was also consistently higher in the experimental crosses at 8.5 and 9.5 days. Although the differences at 8.5 and 9.5 days did not reach statistical significance in Experiment 2, the calculated percentage of "induced" abnormals was close to the predicted 25% and the difference between

Table 2. 1. Frequency of abnormal embryos from control and experimental crosses.

Age (days)	Control cross (*a/m* female x *b/b* male)				Experimental cross (*a/m* female x *b/m* male)				% Induced Small or Abnormal†	Statistical significance§	
	Normal	Small	Abnormal	% Small or Abnormal	Normal	Small	Abnormal	% Small or Abnormal			
Experiment 1: Morphological Study*											
6.5	39	3	1	9.3	27	3	1	12.9	4.0		P=0.25
7.5	51	7	0	12.1	36	19	1	35.7	26.9	χ^2=7.55	P=0.006
8.5	87	6	4	10.3	52	16	9	32.5	24.7	χ^2=13.12	P=0.0003
Total (7.5-9.5)	138	13	4	11.0	88	35	10	33.8	25.7	χ^2=22.16	P=0.0001
Experiment 2: Histological Study											
6.5	18	-	0	0	23	-	1	4.2	4.2		P=0.57
7.5	22	-	2	8.3	40	-	19	32.2	26.0	χ^2=3.98	P=0.047
8.5	7	-	0	0	22	-	9	29.0	29.0		P=0.12
9.5	8	-	0	0	32	-	7	17.9	17.9		P=0.24
Total (7.5-9.5)	37	-	2	5.1	94	-	35	27.1	23.2	χ^2=7.21	P=0.0072

* Data from West *et al*, 1990

† See Materials and Methods for calculation

§ Statistical difference in the proportion of normal embryos between the control and experimental crosses, tested by Fisher's Exact test except when shown as χ^2

the experimental and control crosses was highly significant when data was pooled for 7.5 to 9.5 days. The lack of statistical significance in Experiment 2, at 8.5 and 9.5 days, may simply reflect the small sample size in the control cross; only one litter was used for each of these groups. After correcting for the presence of abnormals in the control groups, the overall frequency of abnormals at 7.5-9.5 days is close to the expected 25%: 25.7% in Experiment 1 and 23.2% in Experiment 2. These frequencies imply that although few, if any, of the homozygous *m/m* embryos were detectable at 6.5 days, most (probably all) were identified by both morphological and histological examination from 7.5 days onwards.

The histological appearance of the embryos in Experiment 2 was studied to try to identify the earliest developmental anomalies in the homozygous mutant embryos. At 6.5 d.p.c. all of the embryos in the control cross appeared normal and had the characteristic elongated egg cylinder appearance (Fig 2.1). In the experimental cross, one of the decidual swellings contained a small necrotic mass but all of the others appeared normal. By 7.5 d.p.c. an abnormal class of embryos were identified in the experimental (*a/m* x *b/m*) cross; 19/59 (32.2%) showed signs of abnormal development, compared with 2/24 (8.3%) embryos in the control cross. A consistent finding in many of the abnormal conceptuses in the experimental cross was that they failed to form the three cavities, normally found in embryos of this age. Normally the amniotic cavity, exocoelom and ectoplacental cavity are separated by the amnion and chorion respectively, as shown in Fig 2.2A.

Two of the 7.5 day abnormal embryos appeared retarded by approximately one day; they were similar to normal 6.5 day embryos although they were slightly smaller. Another embryo (Fig 2.2B) consisted of a solid mass of cells and appeared to have more cells than a normal 6.5 d.p.c. embryo but no systematic cell counts were made. There was an indentation halfway along the length of the embryo, which could be an incomplete amniotic fold. The embryonic ectoderm had not differentiated properly

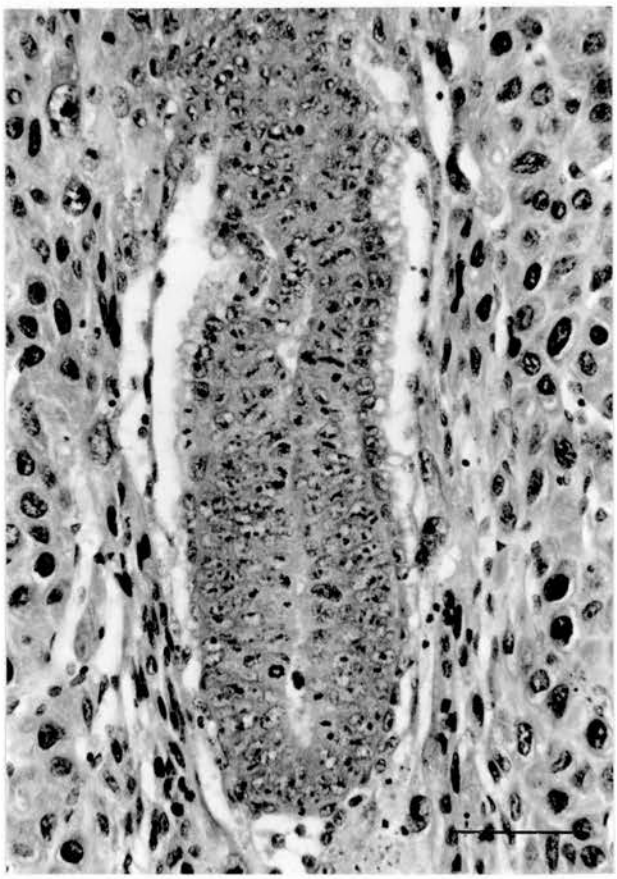


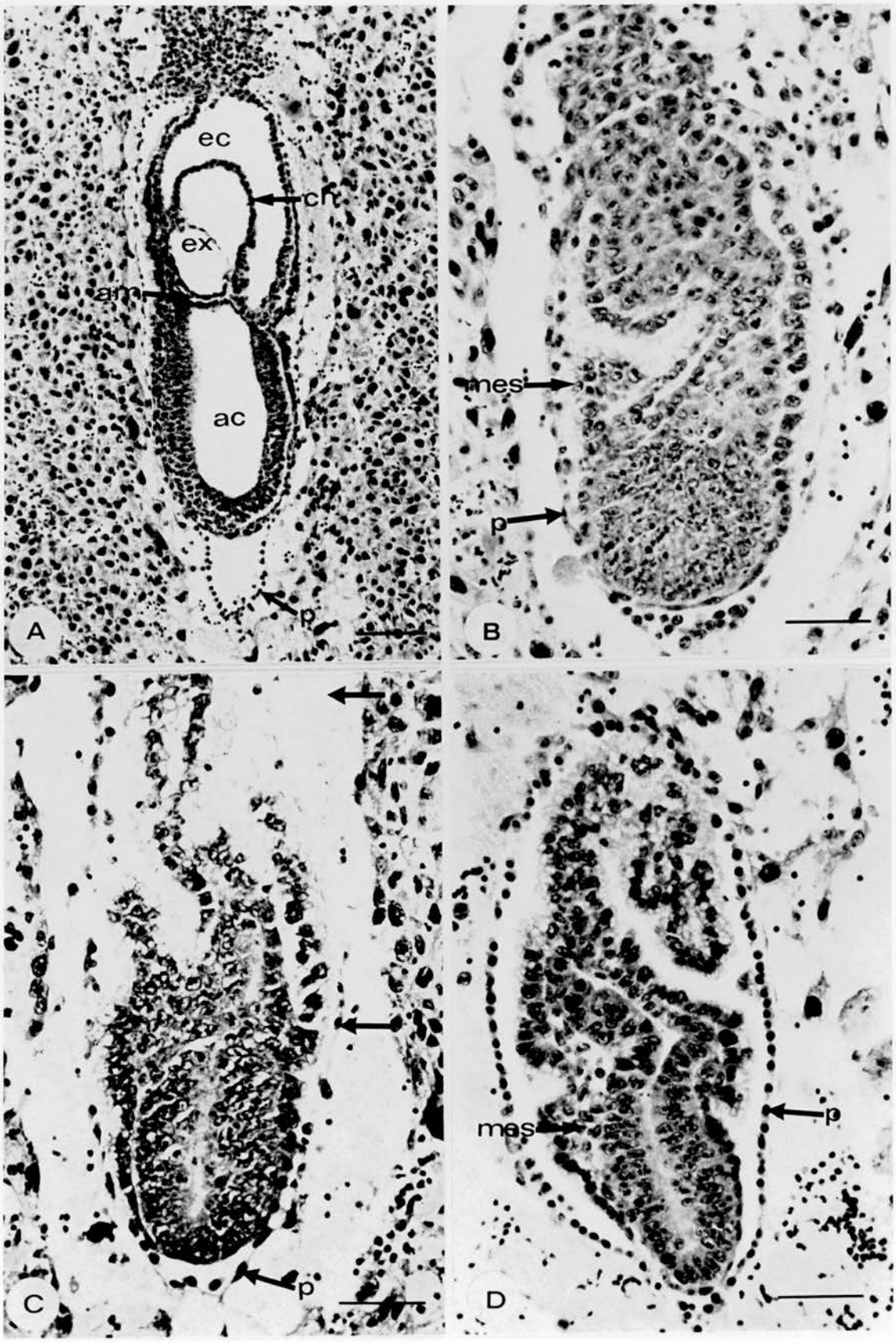
Fig 2.1. A normal 6.5d embryo from the control cross. Scale bar = 50 μm.

and there was no cavity formation. The remainder of the abnormal 7.5 day embryos were deficient in cavity formation and mesoderm production, with some being more advanced than others. A group of three embryos (e.g. Fig 2.2C) had a similar appearance, the embryonic ectoderm had not epithelialised and a small proamniotic cavity had formed in the midline. No mesoderm was present and the extraembryonic part had a pinched appearance as if it has been unable to expand properly and appeared to consist mainly of extraembryonic endoderm. Another conceptus (Fig 2.2D) had a better defined embryonic ectoderm, with a larger proamniotic cavity and a small amount of mesoderm.

At 8.5 d.p.c., all of the embryos in the control cross of Experiment 2 appeared to be normal (Fig 2.3A) and many had begun the turning process (see Kaufman, 1992). Nine out of 31 (29%) embryos from the experimental cross were abnormal and were surrounded by trophoblastic giant cells and blood. Four resembled the 7.5 d.p.c. abnormals, having incomplete cavity formation, little or no mesoderm and a disorganized extraembryonic region (Fig 2.3B). In the remaining abnormal embryos (Fig 2.3C), a small amount of embryonic ectoderm remained and an intermediate layer of mesoderm was present; very little of this mesoderm had spread into the extraembryonic region. The extraembryonic endoderm resembled the yolk sac of normal 8.5 d.p.c. but there was no mesodermal component present. The overall impression of the embryo, shown in Fig 2.3C is that of an expanded egg cylinder and it resembled an expanded version of the 7.5 day embryo shown in Fig 2.2D.

All the embryos appeared normal at 9.5 d.p.c., in the control cross of Experiment 2 (Fig 2.4A). In the experimental cross 7/39 (18%) were abnormal. A few of these resembled the 7.5 day abnormals having a small amount of embryonic ectoderm but with more extensive proliferation of the extraembryonic endoderm (Fig 2.4B). Two embryos were similar to the 8.5 d.p.c. 'expanded egg cylinder' embryos (Fig 2.4C).

Fig 2.2 (A) A normal 7.5 day embryo from the control cross showing typical egg cylinder structure. Three cavities are visible (amniotic cavity, exocoelom and ectoplacental cavity) and these are separated by the amnion and chorion respectively. The embryonic region is below the level of the amnion with the extraembryonic portion above.
(B - D) Abnormal 7.5d embryos from the experimental *a/m* x *b/m* cross showing lack of complete cavity formation and greater proliferation of the extraembryonic part of the conceptus (arrowed in C)
Scale bar = 100μm in A and 50μm in B,C and D.
Key: ac, amniotic cavity; ex, exocoelom; ec, ectoplacental cavity; am, amnion; c, chorion; p, parietal endoderm of Reichert's membrane; mes, mesoderm.


ec
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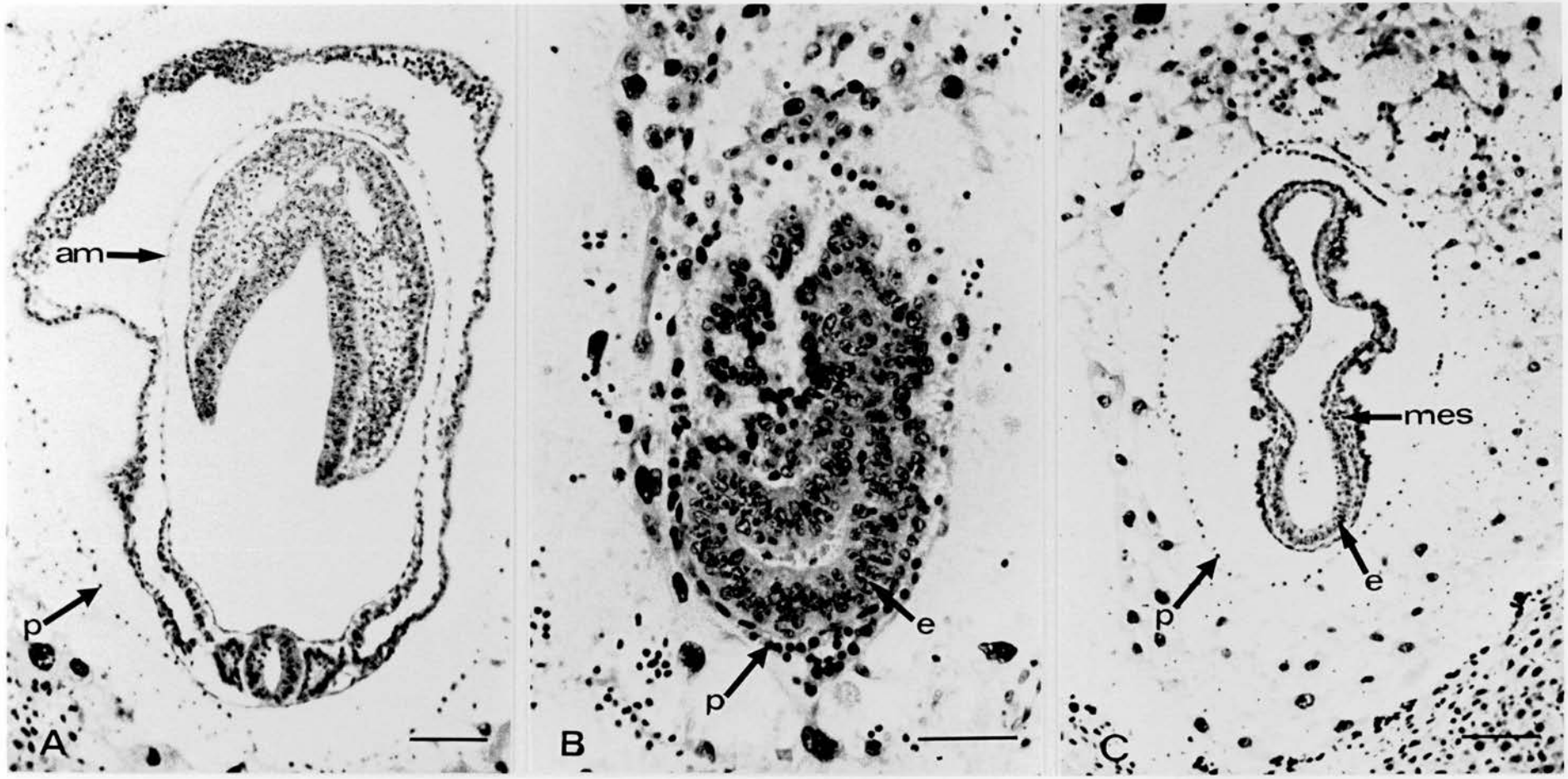
Another two conceptuses contained virtually no embryonic material at all, Reichert's membrane encompassed a large amorphous mass with few cells within (not shown).

2.4 DISCUSSION

The results from the morphological and histological studies both showed that the GPI null embryos appeared normal at 6.5 days but were probably all abnormal by 7.5 d.p.c. This defines 7.5 days as the time when developmental abnormalities, in the homozygous mutant embryos, are first detectable. At this time, the gross morphology of the null embryos appeared retarded compared to normal littermates and the histology shows that the embryos had not formed the cavities normally present at 7.5d and that little or no mesoderm had formed. By 8.5d.p.c the null embryos had progressed little further and now appeared as an expanded egg cylinder with a small amount of embryonic ectoderm and mesoderm remaining. Other than Reichert's membrane (composed entirely of parietal endoderm cells), no extraembryonic membranes had formed because of the lack of mesoderm. The abnormal embryos at 9.5 d.p.c. were very similar to those at 8.5 but, in some cases, Reichert's membrane and the extraembryonic region of the egg cylinder had enlarged. A previous study (West, 1993) showed that 10.5 d.p.c. null embryos resembled the 8.5 and 9.5 day embryos in the present study but by 11.5 and 12.5 d.p.c., only dark staining, necrotic cells within a mass of resorbing tissue remained. Together, the two studies indicate that, apart from expansion of Reichert's membrane and proliferation of the extraembryonic portion of the egg cylinder, the homozygous GPI null embryos appear to progress little between 8.5 and 10.5 d.p.c. and degenerate at 11.5 days.

For development to result in an embryo like that shown in Fig 2.4C, two different pathways could have been followed, that of embryonic loss or embryonic failure (Fig 2.5). In the case of embryonic loss, the embryos would develop normally until at some point, the fetus dies leaving an empty sac like structure consisting of the

Fig. 2.3 (A) An 8.5d normal control embryo.
(B & C) 8.5d abnormal embryos from the experimental cross.
Scale bar = 100μm in A & C and 50μm in B.
Key: am, amnion; e, embryonic ectoderm; p, parietal endoderm.


am
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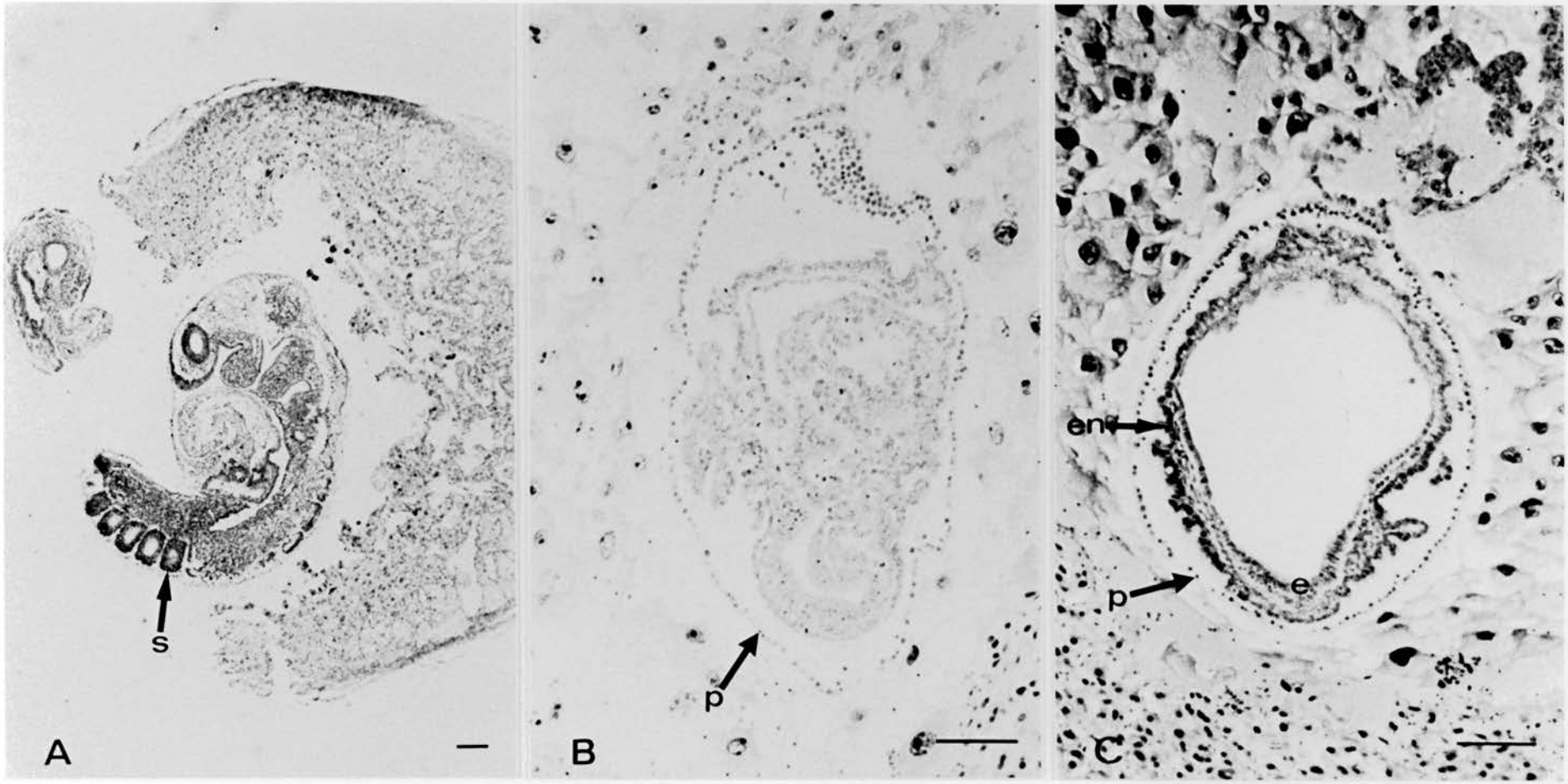
extraembryonic membranes (Fig 2.5 A - D). The present histological study shows that the homozygous *Gpi-1s* null embryos do not form normal fetuses in this way. Instead, the embryo develops normally until at some stage (in this case 6.5 days), abnormalities become apparent and the abnormal embryo continues to expand to form the empty sac structure that is observed (Fig 2.5 a - d). The homozygous GPI null embryos follow the second pathway and therefore the empty sacs found at 9.5 days result from failure of the embryos to develop rather than fetal loss.

The developmental failure in the GPI null embryos occurs around the time of gastrulation. The onset of gastrulation is marked by the appearance of the primitive streak. Cells delaminate from the epithelium of the embryonic ectoderm and migrate towards and through the streak to emerge as mesoderm (Snell & Stevens, 1966). Mesoderm contributes to both embryonic and extraembryonic tissues. At 7.5d.p.c.the mesoderm contributes to both the amnion and chorion which separates the embryo into three compartments and at 8.5 d.p.c the mesoderm contributes to the yolk sac. A failure in this process would result in the loss of mesoderm and its derivative tissues. This appears to be the case in all the abnormal embryos examined from the experimental cross (putative homozygous GPI nulls).

The homozygous null embryos lack GPI which catalyzes the interconversion of glucose-6-phosphate and fructose-6-phosphate. Once the embryos have used up the maternal supply of the enzyme (at approximately 5 to 6 d.p.c.), the normal glycolytic pathway will be blocked (Fig 1.3). Energy is produced in most animal cells by the breakdown of glucose to pyruvate, which is then oxidised via the tricarboxylic acid cycle (TCA). In early postimplantation mouse embryos, the embryo is thought to develop under anaerobic conditions until the placenta forms at around 9.5 d.p.c.(Cox and Gunberg, 1972; Ellington, 1987). Measurements of carbon dioxide and lactate production from glucose in 6-9.5 dp.c. mouse embryos, *in vitro*, indicate that early postimplantation mouse embryos are dependant on glycolysis (Clough and

Fig. 2.4 (A) A normal 9.5 day embryo from the control cross.
(B & C) Abnormal 9.5d embryo from the experimental *a/m* x *b/m* cross.
Scale bar = 200μm in A and 100μm in B & C.
Key: e, embryonic ectoderm; en, extraembryonic endoderm; s, somite.


A
s
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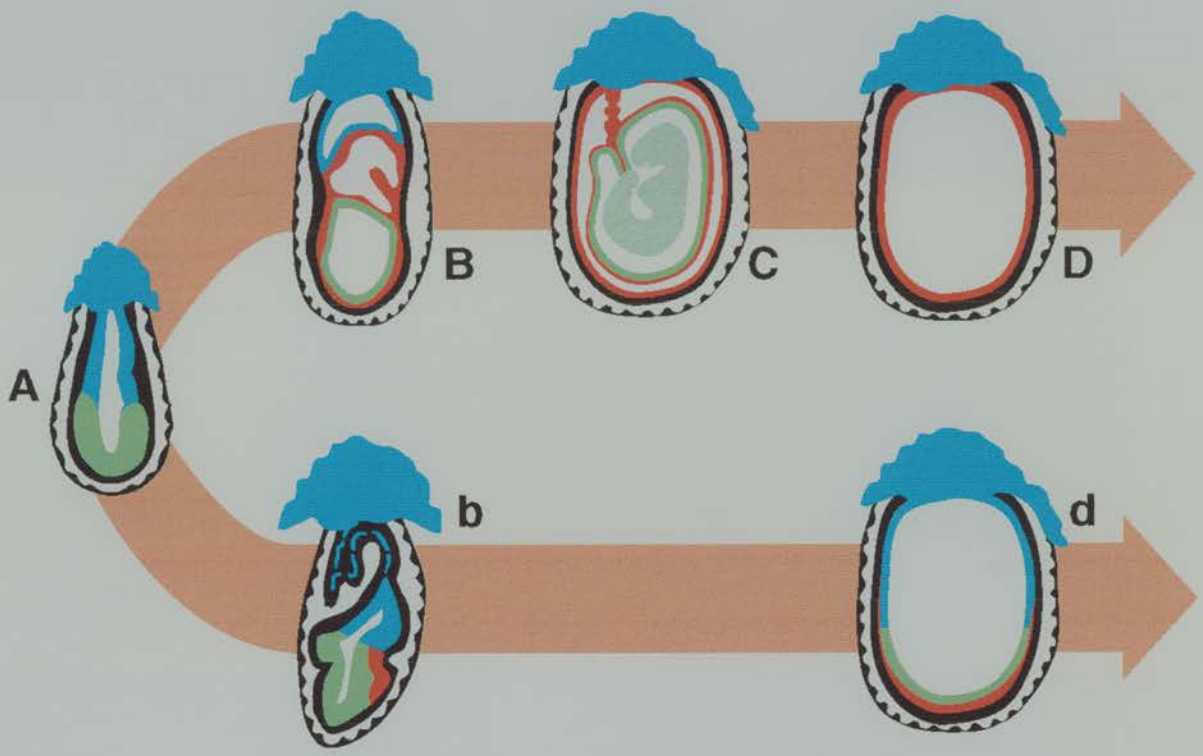
Whittingham, 1983). If the normal route of glycolysis is blocked, as in the case of the homozygous nulls, an alternative source of energy is required. The pentose phosphate shunt could provide an alternative pathway to provide the energy required for normal growth and has been shown to be active at this time (Clough and Whittingham, 1983), but its primary function is to generate reducing power in extramitochondrial cytoplasm in the form of NADPH (Lehninger, 1970). Whatever energy is available from this route may not be sufficient to fuel gastrulation and so the embryo arrests in development at this stage.

Many developmental mutants have been reported that have an effect on the embryo in the early postimplantation period and during gastrulation. The effects of the GPI null mutation are similar to those of Hβ58, an insertional mutation, which interferes with development at the time of gastrulation (Radice *et al* 1991). These embryos are characterized by having small under-developed embryonic ectoderm and proliferation and folding of the extraembryonic membranes. This folding of the extraembryonic membranes is seen in the GPI nulls to such an extent that the cavities in the upper part of the conceptus are unable to form at 7.5 d.p.c.

The null 8.5 and 9.5d abnormal embryos are similar in appearance to 10.5 d tetraploid embryos produced by Tarkowski *et al* (1977). However Tarkowski *et al* reported normal development of the yolk sac whereas the GPI null embryos have only the extraembryonic ectodermal component present, the other component being embryonic ectoderm. Embryos homozygous for the *Brachyury* deletion (*T*) show the first signs of developmental failure at the onset of gastrulation. This locus is first activated in the primitive streak and gene expression continues throughout gastrulation (Beddington *et al* , 1992). Embryos homozygous for the *T* mutation generate insufficient mesoderm but show an increase in the number of embryonic ectoderm cells. They produce normal 8.5day embryos and become grossly abnormal by 9.5 days.

Alternative pathways to embryonic failure



The homozygous GPI null embryos fail at an earlier stage in gastrulation, and it seems more likely that this represents a simple failure in energy supply than, for example, an involvement of glycolysis in activating any genetic locus. This study demonstrates that gastrulation is also dependant on an intact glycolytic pathway.

CHAPTER 3

ATTEMPTS TO CHARACTERIZE THE METABOLIC ACTIVITY IN GPI NULL MUTANT EMBRYOS AND THEIR EXTRAEMBRYONIC MEMBRANES

3.1 INTRODUCTION

In the previous Chapter I showed that a block in the glycolytic pathway due to a lack of GPI causes embryos to arrest at the egg cylinder stage and undergo incomplete gastrulation. Although the homozygous null embryos show signs of abnormal development at 7.5d.p.c. they survive as expanded egg cylinders until about 9.5 d.p.c. before they begin to resorb. During normal embryonic development in the mouse, anaerobic glycolysis is the major energy producing pathway until the placenta forms at about 9.5d.p.c., then the TCA cycle becomes more active (Cox & Guneberg, 1972, Ellington, 1982, Clough & Whittingham, 1983). The survival of GPI null cells at this stage would suggest energy is obtained from an alternative means. It is possible that TCA cycle activity is greater in the outer tissues of wild type conceptuses and in the surviving tissues of the homozygous null conceptuses. To establish whether the TCA cycle was active in the surviving GPI null tissues several preliminary studies were set up. Firstly the likelihood that aerobic conditions necessary for TCA activity were present within the embryos was tested by examining mitochondrial morphology which has been shown to vary with oxygen tension (Morris & New, 1979). Secondly I analysed cytochrome-c oxidase enzymatic activity and mRNA expression. Cytochrome-c oxidase is the terminal enzyme in the electron transport chain which is located on the inner mitochondrial

membrane and it seemed likely that higher levels would be correlated with aerobic type mitochondrial morphology, indicative of TCA cycle activity. Initially normal control embryos were studied but it was planned to extend the study to homozygous null embryos later.

Several predictions of the results were made (Table 3.1). In the control embryos at early post-implantation stages (before the chorioallantoic placenta is established at 9.5 days), energy is produced via anaerobic glycolysis and therefore TCA cycle activity is minimal. Accordingly we would expect reduced levels of enzymes involved in the TCA cycle and the electron transport chain, although active enzyme might persist for some time after synthesis had been down-regulated. The mitochondria should be of the anaerobic type as described by Morris and New (1979) and cytochrome-c oxidase mRNA or enzyme should be absent or detectable only at low levels. The membranes are closest to the maternal environment and may be able to obtain more oxygen than the fetus itself. If so, we would predict some TCA cycle activity in the outer membranes, but anaerobic glycolysis may still predominate. Some mitochondria would therefore be of the aerobic type and cytochrome-c oxidase activity (detected by the assay system or by *in situ* hybridisation) would be higher in the membranes than the embryo.

If the extraembryonic tissues of wild type embryos have a limited TCA cycle potential we would expect this potential to be maximised in the equivalent tissues of the homozygous null conceptuses, that are unable to produce energy by anaerobic glycolysis. If so, more mitochondria would be of the aerobic type and cytochrome oxidase activity should be higher than in wild type membranes.

For reasons described below, none of the approaches investigated were successful in identifying metabolic differences among different tissues or among embryos of different ages.

Table 3.1 Predicted results for early post-implantation stage mouse conceptuses (before placentation established at 9.5 days)

Implantation type	Anaerobic	TCA cycle	Cytochrome oxidase		aerobic
stage	glycolysis mitochondria				
			RNA	Enzyme assay	
Normal (+/+) embryo	++	-	-	-	-
Normal (+/+) membranes	++	±	±	±	±
Homozygous (*m/m*) 'membranes'	-	+	+	+	+

Table 3.2 Predicted results for normal 12.5 day mouse conceptuses (after placenta is established)

	Predicted			Observed†
	O_2 supply	TCA	cyt - c oxidase activity	cyt - c oxidase activity
12.5day embryo	+	+	+	23.5 ± 2.6
outer membrane	++	++	++	25.8 ± 2.5
placenta	+++	+++	+++	30.7 ± 2.1*

* significantly greater than the fetus ($t = 2.62$, $p = 0.0344$)
† see Table 3.3 for results for individual conceptuses.

3.2 MATERIALS AND METHODS

MICE

C57BL/Ola females were mated to C57BL/Ola males to produce a series of control embryos which were used in the mitochondrial morphology study and the cytochrome-c oxidase assay. The mice used for the cytochrome-c oxidase *in situ* hybridisation study from the same cross as those used in section 2.2.

EMBRYO COLLECTION

Females were checked daily for the presence of a vaginal plug. The day of vaginal plug detection was designated as 0.5 days *post coitum* (d.p.c.). Pregnant females were killed by cervical dislocation on 6.5, 7.5, 8.5, and 9.5 d.p.c. for the electron microscopy study and the *in situ* experiment, for the cytochrome-c oxidase assay females were killed on 12.5 and 9.5d.p.c. Embryos within their decidual swelling were dissected free from the uterus in phosphate buffered saline or Hepes buffered M2 medium (Quinn, Barros and Whittingham, 1982) unless otherwise stated.

ELECTRON MICROSCOPY

Transmission electron microscopy was carried out in the Dept. of Anatomy, University of Edinburgh, in collaboration with Prof. M. H. Kaufman and Mr. R. MacDougall.

Embryos within their decidual swelling were dissected free from the uterus and placed in 3% gluteraldehyde (pH7.2) in 0.1 % phosphate buffer. The samples were dissected from their decidual swellings and washed for 30 min in 0.5% sucrose in 0.1% phosphate buffer (pH7.2), then fixed for 1 - 2hrs in 1% osmium tetroxide. This was followed by 2 changes of 10% alcohol for 15 min each, then three 30 min washes in 100% alcohol. The samples were then placed in propylene oxide (BDH)

for 30 min. A stock solution (Sol. A) of DMP30 (2,4,6 - tris (Dimethylaminomethyl) phenol) and dibutyl phthalate (BDH) in a 1:2 ratio was mixed with a 50:50 solution (Sol. B) of DDSA (Dodecenyl Succinic Anhydride) and araldite resin, 19ml of the Sol. B to 0.5ml of Sol. A. The samples were left in this overnight. The next day the samples were removed to fresh Sol. B and heated in a 60°C oven for 48hrs to polymerise. Sections were cut on a microtome (Reichert Ultracut E) at 1μm and stained (see Appendix II) (Ito and Winchester, 1963). Ultrathin sections were then cut at 60-80nm. Because the 9.5d embryos were too large for the whole sample to be examined, after the staining an area was selected for study and the block cut to a size that could be examined. Ultrathin sections were then cut as above. The sections were then immersed in a saturated solution of uranyl acetae in 50% ethanol for 10-15min, rinsed in 50% ethanol and stained with 0.2% lead citrate for 2min and rinsed in distilled H_2O. The sections were viewed at 60 kV on a Philips 301 transmission Electron Microscope.

ENZYME ASSAYS

Cytochrome-c oxidase assays were performed in collaboration with Dr. T. Bramley and Mr. G. Menzies, Dept Obstetrics and Gynaecology, University of Edinburgh.

Initially 12.5day conceptuses were used to test the enzyme assay protocol. It was anticipated that later the techniques could be applied to earlier stage conceptuses. The conceptuses were dissected into 3 parts consisting of the fetus, the placenta and membranes (the amnion and yolk sac). Each sample was weighed and 7ml H_2O added. The sample was then homogenized (using 20 complete strokes) in SET buffer (0.3 M sucrose, 40mM Tris and 1mM EDTA, pH 7.4) using a loose - fitting Dounce homogenizer. Protein was measured by the method of Lowry et al, (1951) using bovine serum albumin (BSA) as a standard.

Cytochrome-c oxidase activity was measured spectrophotometrically by estimating the rate of oxidation of dithionite reduced horse heart cytochrome C at 550nm (20°C). 0.8ml H_2O, 0.1ml cytochrome C (10mg/ml reduced with approximately 10mg of sodium dithionite) and 0.5ml 0.2M Tris-acetate, pH 6.8 were added to a 100µl aliquot of the homogenate (Bramley & Menzies, 1986). The change in O.D. measured on a Sp6 500 UV Pye Unicam spectrophotometer.

PREPARATION OF LABELLED PROBES FOR RNA IN SITU HYBRIDISATION ANALYSIS

RNA *in situ* hybridisation was performed in the MRC Reproductive Biology Unit in collaboration with Dr. Philipa Saunders, Mr. J. Gaughan and Mr. M. Millar.

For preparation of riboprobes, Bluescript plasmid (Stratagene) containing rat cDNA E1 (606bp) was purified and linearized using *Bam* HI and *Hind* III. The template was extracted with phenol/chloroform, precipitated, and dissolved in water; it's concentration was checked on an agarose gel. Labelled sense and antisense cRNA was synthesized by incubation of linearized template with digoxygenin (DIG) - labelled UTP (3.5mmol/L; Boehringer-Mannheim) in the presence of T3 or T7 polymerase (40U; Boehringer-Mannheim) for 2hrs, at 37°C according to manufacturers recommendations (Boehringer-Mannheim). After synthesis, the amount of labelled RNA was determined by comparison to a DIG-labelled RNA standard

CYTOCHROME C OXIDASE RNA IN SITU HYBRIDISATION

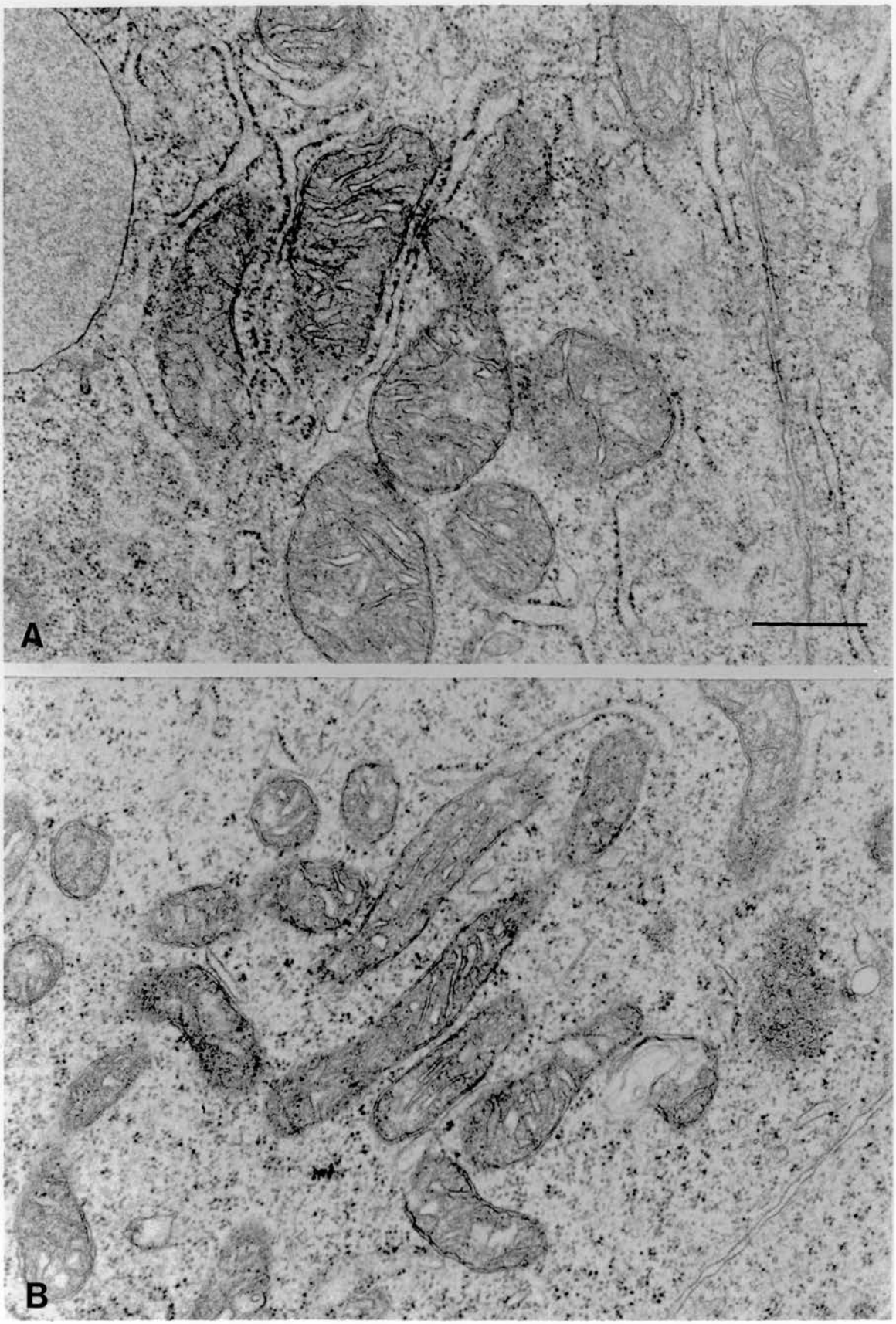
Embryos within their decidual swellings were fixed in Bouins fluid (see Appendix III (b) for) for 5 hr. Embedding of samples and and section thickness are the same as in Chapter 2.2.

Tissue sections were mounted on glass slides coated with 3, aminopropyltriethoxysilane (TESPA, Sigma), dried and dewaxed in xylene for 15min and rehydrated through a series of alcohols. The sections were then treated with acid (0.2 N HCl, 20 min) and partially digested with proteinase K (2μg/ml) for 20 min at 37°C. After incubation with 0.2% glycine for 10 min at 4°C, the sections were acetylated to reduce the electrostatic binding of the probe by acetylating positively charged amino groups, and prehybridized at 50°C for 2hrs. Hybridisation was then performed overnight at 50°C. Excess probe was removed by washing in 2 x SSC at room temperature before ribonuclease A (20μg/ml) treatment. Sections were then washed with 0.1x SSC/30% formamide at 45°C and blocked in normal sheep serum: Tris-buffered saline (1 part sheep serum to 5 parts TBS) for 30 min, drained, and incubated with anti-DIG alkaline phosphate conjugate (Boehringer-Mannheim, 1:300 dilution). Excess antibody was removed by two 15 min washes in TBS (pH7.4), and then sections were equilibrated in TBS (pH9.5) containing 50mM $MgCl_2$ before visualization of tissue mRNA/DIG-cRNA dimers by an enzyme - catalysed colour reaction using 5-bromo-4-chloro-3-indolyl phosphate (X phosphate) and nitroblue tetrazolium salt (see Appendix III (a)).

3.3 RESULTS

ELECTRON MICROSCOPY

A preliminary experiment was undertaken to compare the number, morphology and distribution of mitochondria and number of cristae per mitochondrion in 7.5, 8.5, and 9.5d embryos from control C57BL/Ola matings. Only three 7.5d, one 8.5d and one 9.5 d embryos were examined. The tissues examined were embryonic ectoderm, extraembryonic ectoderm, both embryonic and extraembryonic endoderm, and extraembryonic and embryonic mesoderm. At 7.5d the mitochondria of the embryonic ectoderm were small and numerous compared with those of the other tissues, with mitochondria of the embryonic endoderm being the largest (Fig 3.1A &


A
B

3.1B). The smaller mitochondria had fewer cristae but this may only be a consequence of their smaller membrane area. At 8.5 and 9.5d the fixation protocol was a problem and many of the mitochondria appeared damaged. Insufficient time made continuation of this project impractical.

CYTOCHROME-C OXIDASE ENZYME ASSAY

12.5 day conceptuses were chosen to test the feasibility of the cytochrome-c oxidase assay as they have a well established placenta and were expected to show a high level of TCA cycle activity (see Table 3.2 for the predicted results). The results obtained from the enzyme assay of 8 conceptuses are shown in Table 3.3. The cytochrome-c oxidase activity in the membranes was not significantly different from the fetus or placenta . However, the activity in the placenta was significantly higher than in the fetus (Paired $t=2.62$; $P= 0.0344$).

The 9.5d embryos were dissected and assayed in the same way. However the samples were too small and the assay was not sensitive enough for reliable measurements to be made. The embryos that were of greatest interest i.e. 7.5 - 9.5d homozygous null embryos are considerably smaller than these and therefore no further experiments of this type were undertaken. Instead an attempt was made to compare cytochrome-c oxidase mRNA levels by RNA *in situ* hybridisation.

CYTOCHROME C OXIDASE RNA IN SITU HYBRIDISATION

Cytochrome-c oxidase has thirteen subunits, three of which (I - III) are coded for by mitochondrial DNA. Cytochrome oxidase II (COXII) mRNA encodes all or part of the second subunit of cytochrome-c oxidase (Grosskopf & Feldman 1981 and Brown & Simpson 1982) The cellular distribution and pattern of cytochrome oxidase II (COXII) mRNA expression was investigated using *in situ* hybridization with a nonradioactive probe. The *in situ* technique employed here can detect COX II mRNA in the basal area of rat seminiferous epithelium and predominantly in

Table 3.3. 12.5 day C57BL/Ola embryos cytochrome - c oxidase assay
Cytochrome-c oxidase activity measured as change in OD/min/mg protein

Conceptus	Fetus	Membranes	Placenta
1	20.7	19.6	34.9
2	23.8	33.3	27.8
3	23.8	26.9	31.2
4	15.8	13.5	24.8
5	20.5	31.0	23.8
6	37.5	28.5	39.7
7	29.8	32.6	26.0
8	16.1	21.2	37.4
Mean, ± S.E.	23.5 ± 2 .6	25.8 ± 2.5	30.7 ± 2.1

pachytene spermatocytes (Saunders *et al* 1993). We wanted to investigate whether it could detect COX II mRNA in different areas of mouse conceptuses. The conditions in the original protocol (Saunders *et al*, 1993) were not optimal for mouse tissue. The original 40 min proteinase K digestion time was found to be too severe for the mouse tissue, stripping almost everything off the slides. A control experiment was run consisting of rat and mouse testis with a proteinase K digestion step of 5 - 30 min, (5, 10, 15, 20, 25, or 30 min) to try to find the most favourable treatment time and also to check that the rat probe would pick up COXII mRNA in mouse tissue. A positive result in control mouse testis tissue was found at 20 min. In the rat testis, the pachytene spermatocytes show a high level of expression (Fig 3.2A) but in the mouse testis, elongating spermatids and the cells around base of the tubules appeared to be positively stained (Fig 3.2B). This result may not be characteristic of the mouse testis as the corresponding rat control was not totally convincing. Several attempts were made to analyse cytochrome-c mRNA in mouse embryo sections but for various technical reasons, none were successful. Due to a lack of time this study was not taken any further.

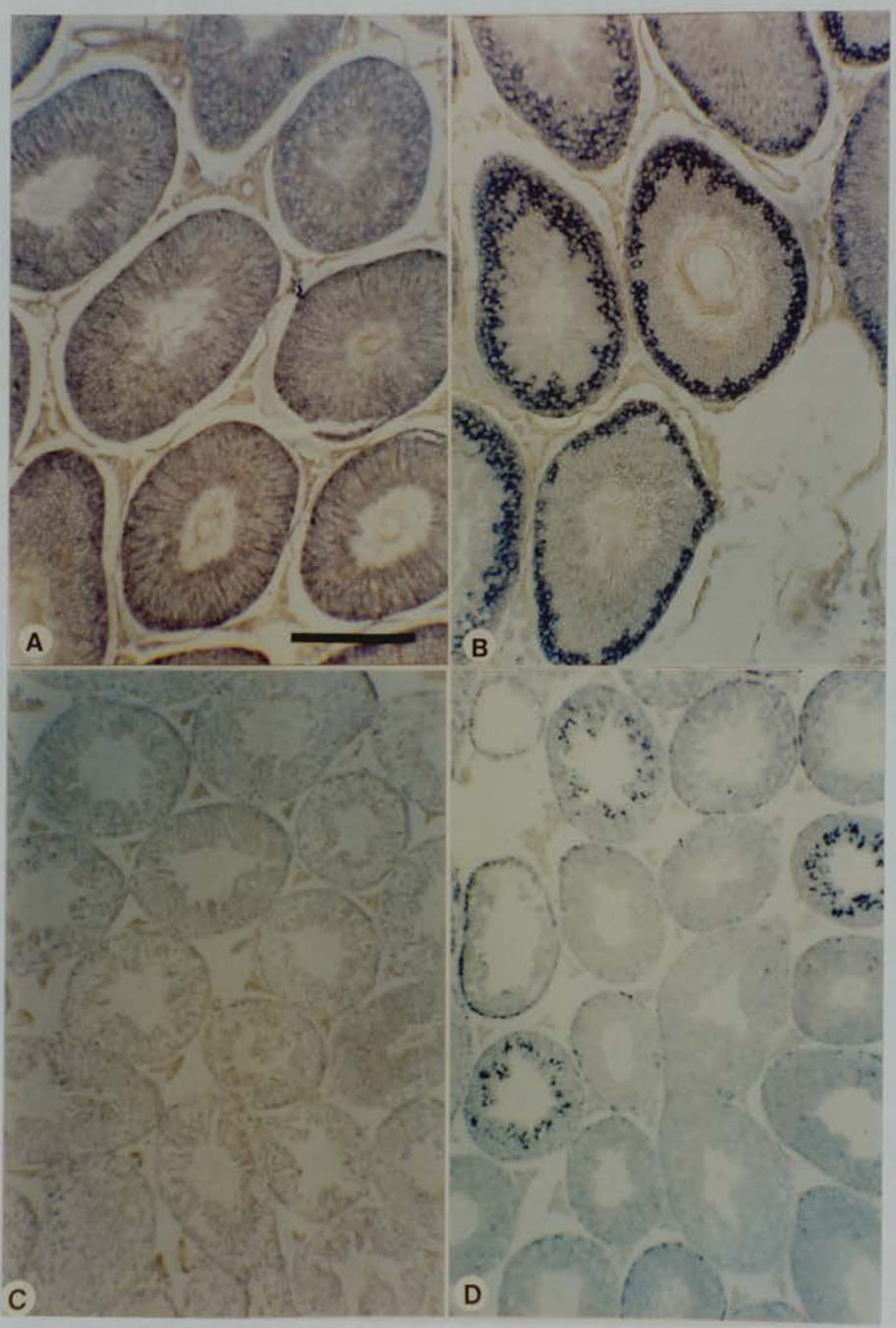
3.4 DISCUSSION

The predicted results for the mitochondrial study (Table 3.1) were that mitochondria of embryos 7.5-9.5d.p.c. should have been of the anaerobic type but that at least some of the mitochondria of the extraembryonic membranes should be aerobic if they had access to the oxygen present at the fetal/maternal interface. Unfortunately we were unable to substantiate this prediction. Preliminary results of the study of mitochondrial morphology suggested that in the normal 7.5 d embryos the embryonic ectoderm had more numerous, small mitochondria present with the extraembryonic endoderm having larger mitochondria. Many technical problems were encountered in quantifying results. The fixation of the mitochondria in the larger 8.5 and 9.5d embryos was poor and many of them appeared to contain large

Fig 3.2 A & B Cytochrome oxidase *in situ* hybridisation of (A & B) rat testis labelled with (A)Bam H1 and (B) HindIII, showing hybridisation in pachytene spermatocytes.

Fig 3.2 C & D Mouse testis labelled with (C) BamH1 and (D) Hind III showing hybridisation in elongating spermatids and cells around the base of tubules.

Scale bar = 500μm


A
B
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D

spaces. It is not clear whether this was a fixation artefact or reflected genuine differences in their metabolic activity. A mitochondrion with no cristae could just be one that has been damaged during the processing. In our preliminary observations it was difficult to quantify number of cristae and compare sizes of mitochondria because these characteristics are dependant on the plane of section. What appears to be a small round mitochondrion could in fact be a long tube-shaped one. To find out which of these is true, serial sections through the mitochondrion would have to be examined, following it through the different sections to work out the actual shape. The difficulties encountered while investigating the control embryos persuaded us to abandon the intended study of the distribution of mitochondrion and cristae in the null embryos. This would have entailed an extensive study and there was insufficient time.

The cytochrome-c oxidase enzyme assay was initially carried out on 12.5 day old conceptuses. It was predicted that the TCA cycle would be more active in the placenta than either the fetus or the outer membranes because average oxygen levels would be higher in the presence of oxygenated maternal blood (Table 3.2). The results of the assay confirmed this prediction. The cytochrome-c oxidase activity in the fetus and placenta was significantly different but there was no difference between the fetus and membranes. The assay was not sufficiently sensitive for individual 9.5day embryos. The homozygous null embryos are much smaller than normal 9.5 day embryos. Therefore conceptuses from several litters would have to be collected to provide enough material for the assay. Such a large scale embryo collection was not thought feasible in the time available.

The cytochrome-c oxidase *in situ* hybridisation protocol followed had been optimized for conditions in the rat testis. Unfortunately these conditions were not suitable for mouse tissue and further time would have been necessary to optimize the protocol. I found a pattern of staining in the mouse testis that differed from the rat.

This could be an actual species difference but it may be due to an artefact of the proteinase K digestion step destroying so much of the tissue that the probe hybridized non specifically. The experiments involving embryonic tissue failed technically. Time did not permit further follow up to this study, but it would be worthwhile to try to overcome the technical problems encountered.

Recent work has shown that there is no significant difference in the pattern of carbohydrate metabolism in different tissues of normal embryos (H. Leese personal communication). It would however be interesting to continue to study the metabolism of the homozygous null embryos to see whether the same is true or if the activity of the abnormal embryos differs from that of the membranes.

CHAPTER 4

COMPARISON OF THE GPI NULL AND CBA/J X DBA/2 MODELS OF EMBRYONIC LOSS.

4.1 INTRODUCTION

In mice approximately 10% of embryos die *in utero* (Leonard et al 1971). Clark *et al.*, (1980) found that in CBA/J females mated to DBA/2 males, the frequency of embryonic resorptions was higher (~30%) than in matings with C3H/HeJ males bearing the same H2 haplotype (H-2d) as DBA/2. The resorption frequency in these (CBA/J x DBA/2) matings, varied from one breeding centre to another (Chaouat, 1989). The high frequency of resorptions in the cross were thought to have an immunological basis and to be influenced by the environment in which the animals were maintained. Microbial and viral pathogens were assumed to be the cause of the high incidence of resorptions seen (Hamilton and Hamilton, 1987), but more recently a study by Baines *et al*, 1994 suggests that pathogens may not be the cause of the resorptions observed.

At present, cytokines such as Tumour Necrosis Factor alpha (TNF-α) and cells of the innate (natural) immune system, such as macrophages and Natural Killer (NK) cells are thought to play a role in murine abortion. The predominant suppressive molecule at the murine feto-maternal interface is a transforming growth factor-β_2 (TGF-β_2) related molecule (Lea et al, 1992). The TGF-β_2 like factor is released by a sub-population of small sized trophoblast-dependant cells present in the decidua basalis and the metrial gland area. Low levels of suppression of this factor is associated with abortion (Clark *et al.*, 1991). The (CBA/J x DBA/2) pregnancies

have been associated with a delayed release of the TGF-β_2 related suppressor factor (Day 10.5) at the implantation site with respect to TNF-α (Day 8.5). It is during this time that the majority of abortions occur (Clark *et al*, 1991). Early in development at 6.5 -7.5 days an infiltrate of NK cells is detected (Gendron & Baines, 1988) in those implants destined to abort, then at 9.5 - 11.5 d.p.c an infiltrate of polymorphonuclear leukocytes (PMN) is detected in the resorbing conceptuses (Gendron & Baines, 1989). This contrasts with control, low aborting pregnancies (C3H/HeJ x DBA/2) where decidual TGF-β_2 and TNF-α arise simultaneously at 8.5d.p.c. (Clark *et al*, 1991). Furthermore, TNF-α levels are reported to be higher in the placentae of DBA/2 mated CBA/J females and the injection of TNF-α increases the resorption rate in abortion-prone (CBA/J x DBA/2) and non abortion-prone murine pregnancies (Tangri & Raghupathy, 1993; Chaouat *et al*, 1990).

Although a great amount of work has centred on the immunological mechanisms involved in the resorption of the (CBA/J x DBA/2) fetuses as a model for recurring spontaneous abortion in humans, there have been few reports on histological appearance of the resorbing embryos. In this study the morphological characteristics associated with embryonic lethality of the DBA/J x CBA/2 system will be compared with those seen in the homozygous GPI (*m/m*) null embryos (Chapter 2). Additionally an *in situ* hybridisation study will look for the presence of TNF-α mRNA in the normal and resorbing CBA/J fetuses and its expression will be compared to that of normal, heterozygous and abnormal, homozygous GPI null embryos.

Delayed expression of TGF-β_2 and the presence of TNF-α (in the absence of TGF-β_2) is unlikely to be a primary **cause** of embryonic death of the homozygous GPI nulls, which almost certainly die as a direct consequence of the glycolytic deficiency caused by the lack of GPI (Chapter 2). However inappropriate expression of TGF- β_2 and/or TNF-α may occur as a **consequence** of the developmental failure of the

homozygous null embryo. If so, it may elicit the eventual resorption of the expanded egg cylinder structure. Analysis of the expression of TNF-α in homozygous GPI null embryos may determine whether abnormal expression is a cause or consequence of the embryonic death of the (CBA/J x DBA/2) embryos. Even if there is no involvement of these factors it will still be of interest to compare the morphological characteristics of both types of lethal embryos.

However, in this study, because the *in situ* hybridisation technique had not been optimised, a comparison of the expression of TNF-α in the two models could not be made. Therefore it was not possible to determine whether abnormal TNF-α expression occurred in GPI null embryos and so it could not be determined whether the previously observed abnormal expression of TNF-α in the (CBA/J x DBA/2) embryos was a cause or a consequence of the embryonic loss.

4.2 MATERIALS AND METHODS

MICE

DBA/2 males and the foundation stock of CBA/J mice were obtained from Harlan Olac, U.K and BALB/c mice were obtained from the Dept. of Medical Microbiology, University of Edinburgh. CBA/J is H-2 haplotype, H-2k whereas both DBA/2 and BALB/c are H-2d (Green, 1981). Histological sections of the eyes were prepared from several CBA/J animals to ensure they carried the retinal degeneration (*rd*) allele, which is characteristic of this strain. In the experimental matings, CBA/J females were crossed with DBA/2 males, and control matings, the CBA/J females were mated with BALB/c males. The female reproductive tract was of CBA/J genotype in both the experimental and control crosses, and both crosses were H-2k x H-2d. In addition to the allogeneic pregnancies, syngeneic pregnancies were set up by crossing CBA/J females to CBA/J males to compare the types of abnormalities found in this cross to the allogeneic pregnancies. The GPI homozygous null mice

used in the comparative *in situ* hybridisation study were from the same cross as those used in section 2.2.

EMBRYO COLLECTION

Females were checked daily for the presence of a vaginal plug. The day of vaginal plug detection was designated as 0.5 days post coitum (d.p.c.) as mating was assumed to have occured the preceeding night. Pregnant females were killed by cervical dislocation on 9.5 - 13.5 d.p.c. depending on the study. Females used in the morphology study were sacrificed on day 11.5 - 13.5, and for the histological study on day 9.5 - 12.5. Embryos within their decidual swelling were dissected free from the uterus in phosphate buffered saline or Hepes buffered M2 medium (Quinn, Barros and Whittingham, 1982). For in situ hybridisation, uteri from pregnant females were dissected out on 10.5 and 12.5 d.p.c. After fixation each embryo was dissected in half within the uterus in the same buffers stated above before processing.

MORPHOLOGY

Embryos were classified as normal, small or resorbing. No rigid criteria for classification was used; comparison was made to other embryos in the same litter

HISTOLOGY

Embryos and decidual swellings were fixed in 3:1 ethanol:acetic acid overnight, The samples were then passed through a graded series of ethanol, followed by HistoClear (National Diagnostic) in order to dehydrate the samples and then immersion in 50:50 Histo-Clear:paraffin wax mixture, before paraffin wax under vacuum. After processing the samples were embedded in wax and stored at 4°C. Sections of the tissue were cut at 7μm. The sections for the histological study were stained with haematoxylin and eosin.

IN SITU HYBRIDISATION

The *in situ* hybridisation was carried out in collaboration with Dr. R. Lea in the Metabolic Unit, Dept. of Medicine, University of Edinburgh, Western General Hospital.

Pregnant uteri were fixed in either 4% paraformaldehyde at 4°C overnight (maximum period of fixation = 24hr) or PLP (see Appendix III (b)) and processed as for histology.

Seven micron sections were cut and floated onto TESPA coated slides and dried at 60°C overnight. Sections were then de-waxed in 2 washes of xylene, rehydrated through a series of alcohols (100%, 95%, 70%) and soaked in 2 x SSC for 10 mins. The slides were then immersed in 0.05% protease for 2.5 mins (37°C) and post fixed in 4% paraformaldehyde for 10 mins at 4°C. After treatment the slides were immersed in freshly prepared 0.25% acetic anhydride in 0.1M triethanolamine buffer [pH8.0] for acetylation followed by 10 mins in 0.1 M glycine, in 0.1M Tris-HCl [pH7.4]. Following a brief wash in 2 x SSC the slides were then dehydrated in 70%, 95% and 100% ethanol and air dried. Hybridization was carried out at 48°C and the coverslips were sealed with rubber cement. The hybridization solution contained the following: ^{35}S - labelled TNF-a cDNA probe or a 1kb ladder (5 x 105 c.p.m/30ml), 2 x SSC, 50% formamide, 0.5 x Denhardt's solution, 10mM DTT, denatured salmon sperm DNA (1mg/ml) and yeast tRNA (0.1mg/ml). An aliquot (30ml) of this solution was applied to each slide and the hybridisations were allowed to proceed at 48°C for 14 - 16 h. The slides were then washed in 1 x SSC/50% formamide at 42°Cfor 30 min followed by 0.1 x SSC for 10 min at room temperature. Finally the slides were dipped in nuclear track K5 emulsion (Ilford Limited, Mobberely, Cheshire, UK) diluted with an equal volume of distilled water dried in the upright position overnight and transferred to a light sealed box for 10 - 14 days. After development using Kodak D-19 developer, the slides were counterstained with

haematoxylin and eosin and examined by water and oil immersion light microscopy as appropriate.

4.3 RESULTS

The aim of the experiments described in this Chapter was to compare two models of embryonic loss. The histology of the resorbing embryos from these two models was compared. If the homozygous GPI null embryos (which die because of a glycolytic deficiency) show the same level of TNF-α expression as reported for (CBA/J x DBA/2) pregnancies, this would imply that these abnormalities are a consequence rather than a cause of embryonic death. Conceptuses from different crosses were produced and analysed in different ways. First, specimens were produced for histology and mRNA analysis. Second, additional matings were set up to increase the number of conceptuses in each group so that the frequency of resorbing conceptuses could be compared. The results are presented in the more logical order of morphology, histology and mRNA *in situ* hybridisation rather than the true chronological order.

GROSS MORPHOLOGY

Table 4.1A shows the results obtained from a similar study by Baines *et al* 1994 which was used as a reference study to determine which stages to investigate and also suitable control matings (Table 4.1B). According to the Baines study, the largest increase of resorbing fetuses becomes apparent at 11.5 days. In the present study embryos were dissected free from the uterus at 11.5, 12.5 and 13.5 d.p.c. A comparison between littermates was made to classify them as either normal, small or resorbing. The results are shown in Table 4.2. At 11.5 days, only 5/90 (5.6%) of the conceptuses recovered from the experimental (CBA/J x DBA/2) matings were resorbing, also 2 of the embryos were slightly smaller than their littermates. Even if the 2 smaller embryos were included in the resorbing embryo class, the frequency of

Table 4.1A Data taken from Baines *et al* 1994. Embryo implantations and loss during CBA/J x DBA/2 murine gestation.

Days post coitum	No. of CBA/J female mice	Mean no. of embryos per uterus	% Embryos resorbed
8.5	10	9.8 ± 2.1	Not detectable
9.5	9	10.0 ± 1.6	5.5 ± 6.6
10.5	11	10.4 ± 1.7	15.3 ± 10.0
11.5	40	7.6 ± 2.4	22.9 ± 17.3
12.5	23	8.1 ± 2.6	23.3 ± 14.0
13.5	16	7.4 ± 2.5	24.6 ± 24.0
14.5	8	5.7 ± 0.8	8.5 ± 11.9
15.5	14	7.2 ± 2.1	7.5 ± 14.7
16.5	14	7.6 ± 2.5	14.9 ± 16.8
17.5	8	7.7 ± 2.1	4.3 ± 6.0
18.5	4	8.8 ± 0.8	2.2 ± 4.4
19.5	4	7.3 ± 2.5	10.3 ± 15.7

Table 4.1B Data taken from Baines *et al*, 1994. Embryo losses for CBA/J females mated by syngeneic or allogeneic males

Female x male mating pair	No. of mice	Embryos per uterus (day 12.5 of gestation)	% Embryos resorbed
CBA/J x DBA/2	39	7.8±2.3	23.8±15.4
CBA/J x BALB/C	12	5.6±1.4	5.3±7.8
CBA/J x CBA/J	12	6.0±0.6	5.2±7.3
CBA/J X CFW	12	9.3±1.1	2.6±6.9

Data are presented as the mean and standard deviation.

resorption would only increase to 7.8%. At this stage a similar number of resorbing conceptuses were found in the control cross, 5/95 (5.3%). A day later when Baines and colleagues still found a high proportion of resorbing embryos (Table 4.1), our experimental cross showed only 8.2% of the 98 embryos recovered to be resorbing with only 1 appearing slightly smaller than the littermates, the number of resorptions observed in the control BALB/c cross was similar being 10/99 (10.1%), with 3 appearing slightly smaller than littermates. In the experimental cross at 13.5d.p.c. again only 5 of 98 embryos were resorbing (5.1%) and by this stage the resorptions were almost complete with only a small amount of tissue remaining. In the control cross at this stage again an equivalent proportion of the embryos were resorbing (8.9%). The number of resorptions observed in both the control and experimental groups are very similar and do not exceed the 10% value for normal matings. On the basis of these results it would appear that the resorption frequency in our (CBA/J x DBA/2) matings is not higher than normal which conflicts with previously published results (Clark, *et al* 1980, Chaout, 1989, and Baines *et al*, 1994). However from our results from the histological study (below) were more in keeping with previous studies.

HISTOLOGY

A major objective for the study was to examine the morphology in histological section of resorbing (CBA/J x DBA/2) embryos. A second series of crosses were set up and the development of embryos from one or two representative pregnancies from the experimental and control crosses were assessed. Table 4.3 summarises these results.

Table 4.2 Summary of normal and resorbing embryos recovered from CBA/J females mated to either DBA/2 or BALB/C males.

Mating combination (CBA/J x DBA/2)					
Age	No. of pregnant females	No. of implantations	Normal	Small	Resorb (%)
11.5	10	90	75	2	5 (5.6)
12.5	10	98	90	0	8 (8.2)
13.5	14	98	93	1	5 (5.1)
Mating combination CBA/J x BALB/C					
Age	No. of pregnant females	No. of implantations	Normal	Small	Resorb (%)
11.5	12	95	75	9	5 (5.3%)
12.5	14	99	86	3	10 (10.1)
13.5	15	101	90	2	9 (8.9)

Table 4.3 The number of normal and abnormal (%) embryos observed from each cross at the different ages analysed in the histological study.

Age	Experimental cross (CBA/J x DBA/2)		Control cross (CBA/J x BALB/C)		Syngeneic cross (CBA/J x CBA/J)	
	Normal	Abnormal (%)	Normal	Abnormal (%)	Normal	Abnormal (%)
9.5	22	6 (21.4)	6	2 (25.0)	5	4 (44.4)
10.5	20	2 (9.8)	14	1 (6.7)	11	6 (35.5)
11.5	21	6 (22.2)	20	0 (0.0)	13	5 (27.8)
12.5	21	6 (22.2)	15	3 (16.7)	16	3 (15.8)
Total	84	20 (19.2)	55	6 (9.8)	45	18 (28.6)

a) Frequency of Abnormal Conceptuses

Unlike the series shown in Table 4.2, overall the experimental crosses shown in Table 4.3 did produce more abnormal conceptuses than the control cross (20/104 versus 6/61) but the difference is not statistically significant (χ^2= 1.898, P=0.168).

At 9.5 days the proportion of abnormal embryos in the experimental and control crosses were similar i.e. 21.4% (6/28) and 25% (2/8) respectively, but few control crosses were analysed by histology.

At 10.5 d.p.c., only 9.8% of the experimental embryos examined were abnormal compared to 6.7% in the controls.

By 11.5 d.p.c., 22.2 % of the embryos examined in the experimental cross were resorbing however no resorptions were observed in the controls.

At 12.5 d.p.c., the frequency of resorptions was 6/27 (22.2%) in the experimental cross and 3/18 (16.7%) in the control cross.

The overall frequency of resorptions (these include the abnormal conceptuses which probably would also resorb) in the experimental (CBA/J x DBA/2) cross is 19.2%. This value is greater than would be expected if the resorptions were occuring sporadically but is not statistically significantly greater than the overall total of 9.8% seen in the controls (χ^2= 1.898, P=0.168). Within the (CBA/J x DBA/2) cross the frequency of resorption are similar for the 9.5, 11.5 and 12.5 age groups, however the day 10.5 results were lower (9.8%). In the control CBA/J x BALB/c pregnancies, the resorption rates varied between the age groups, however the overall resorption rate of 9.8 % is as expected for a normal murine cross with only spontaneous resorptions. The only significant difference between the age groups studied in the experimental and control cross was at 11.5d.p.c (Fishers exact test, P=0.028), when

the number of resorptions in the experimental cross was significantly higher than in the control cross.

Comparison of the experimental (CBA/J x DBA/2) and control (CBA/J x BALB/c) crosses with a syngeneic (CBA/J x CBA/J) cross (Table 4.3) revealed the highest frequency of abnormal embryos in the (CBA/J x CBA/J) cross. The overall frequency of abnormals in this cross (18/63) was not significantly higher than that in the experimental (CBA/J x DBA/2) cross (χ^2=1.948, P=0.1629) but was significantly higher than in the control (CBA/J x BALB/C) cross (χ^2=6.970, P=0.0083).

b) Histological Appearance of Abnormal Conceptuses

At 9.5d.p.c., the 6 abnormal (CBA/J x DBA/2) embryos observed were all from the same litter. Five of these had a similar appearance, the embryo was retarded by approximately two days and looked as if it had been tilted within the decidual swelling (Fig 4.1A). The remaining abnormal conceptus was surrounded by blood and small infiltrating lymphocytes with only a small fragment of necrotic embryonic material remaining and was classed as resorbing (Fig 4.1B). The remaining embryos in this cross were retarded by approximately 1- 1.5 days, as none of them had turned completely. In all of the conceptuses examined there was an infiltrate of lymphocytes (Fig 4.1A & Fig 4.1B), and around the fetal maternal interface were what appeared to be dying, degenerating trophoblast giant cells which were darkly stained. In the (CBA/J x BALB/c) group, 2 out of the 8 embryos collected were abnormal. The decidual swellings had a strange shape. The placenta and Reichert's membrane were present but there was no embryo or extraembryonic membranes (Fig 4.1C). The developmental stage of the normal embryos was slightly behind what is expected at 9.5 d.p.c., only one of the embryos had turned and the remaining 5 were retarded by approximately 1-1.5 days. Again an infiltrate of cells and dead trophoblast cells were observed. In the (CBA/J x CBA/J) syngeneic cross (day 9.5),

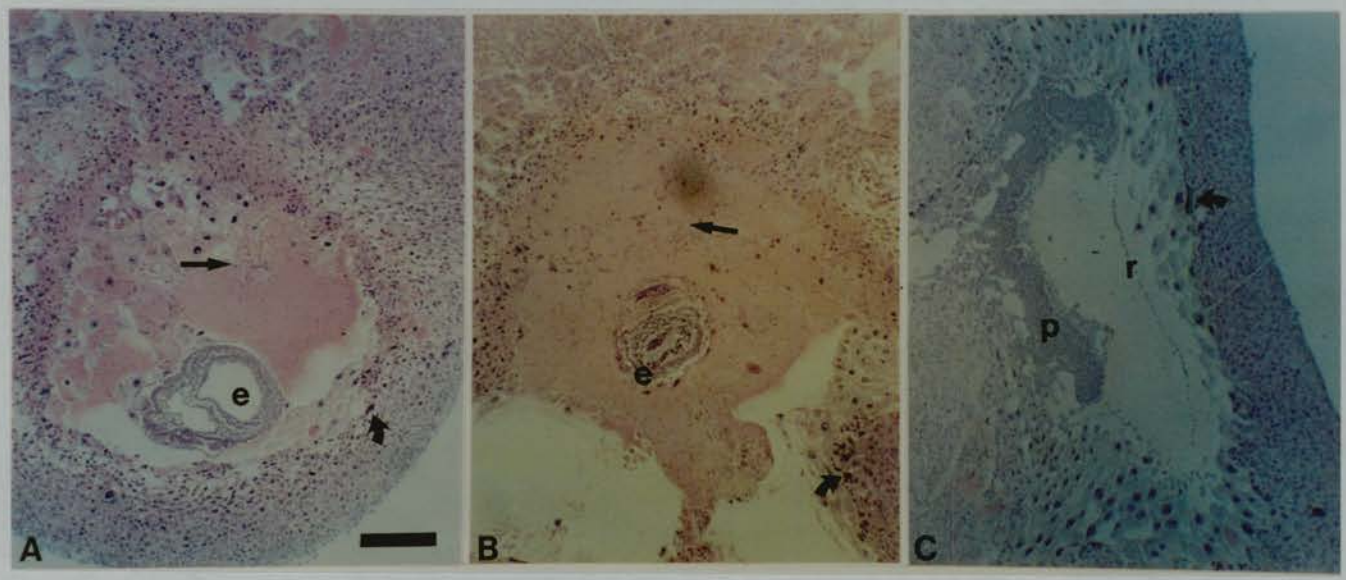


Fig 4.1A. 9.5 day CBA/J x DBA/2. The embryo resembles a 7.5 day old embryo and appears to have tilted within the decidual swelling.
Fig 4.1B. Resorbing 9.5 day CBA/J x DBA/2 conceptus.
Fig 4.1C. 9.5 day CBA/J x BALB/c 'empty sac' conceptus consisitng of placenta and Reichert's membrane.
Scale bar = 250mm
e = embryo, p = placenta, r = Reichert's membrane, small arrow = infiltrate, large arrow = darkly staining trophoblast giant cells.

only 9 embryos were examined and 4 of these were abnormal. One was similar to the (CBA/J x DBA/2) conceptus shown in Fig 4.1A, being retarded and tilted within the decidua and another was similar to the (CBA/J x BALB/c) abnormal conceptus shown in Fig 4.1C. The remaining 2 abnormal conceptuses at this stage had no recognizable embryonic material remaining within the decidua. Of the 5 remaining embryos examined only one had turned and the other 4 were similar to 8 - 8.5d.p.c.

At 10.5 d.p.c. in the experimental (CBA/J x DBA/2) cross, one of the abnormal embryos consisted of what appeared to be a disorganized yolk sac, folded up on itself and surrounded by Reichert's membrane. A small amount of placental tissue was still present (Fig 4.2A). The other abnormal embryo at this stage was retarded by approximately 2 days and looked like an unturned 8 - 8.5 day embryo (Fig.4.2B). The one abnormal (CBA/J x BALB/c) conceptus at this stage, was a mole with no identifiable embryonic material (Fig 4.2C). In the day 10.5, (CBA/J x CBA/J) cross, the 6 abnormal embryos were all from the same litter. Five were moles similar to that shown in Fig 4.2C, the embryos had almost completely resorbed. The remaining abnormal conceptus in this cross consisted of what appears to be placenta and Reichert's membrane with the necrotic remains of a resorbing embryo within (Fig 4.2D).

At 11.5 d.p.c in the experimental (CBA/J x DBA/2) cross, the 6 abnormal embryos were all very similar in appearance. It looked as if the embryo had formed but was degenerating. There were varying degrees of survival of the structures present. In some only a small part of the dying embryo remained, whereas in others there was a larger remnant and these were similar to the abnormal 10.5 day embryo seen in Fig 4.2D. In two of them, the yolk sac and amnion were present as well as the placenta and Reichert's membrane (Fig 4.3A). In the (CBA/J x BALB/c) control however, all of the 20, day 11.5 embryos examined were normal. In contrast, 5 abnormal embryos were found in the (CBA/J x CBA/J) syngeneic cross. Four of these were moles, and

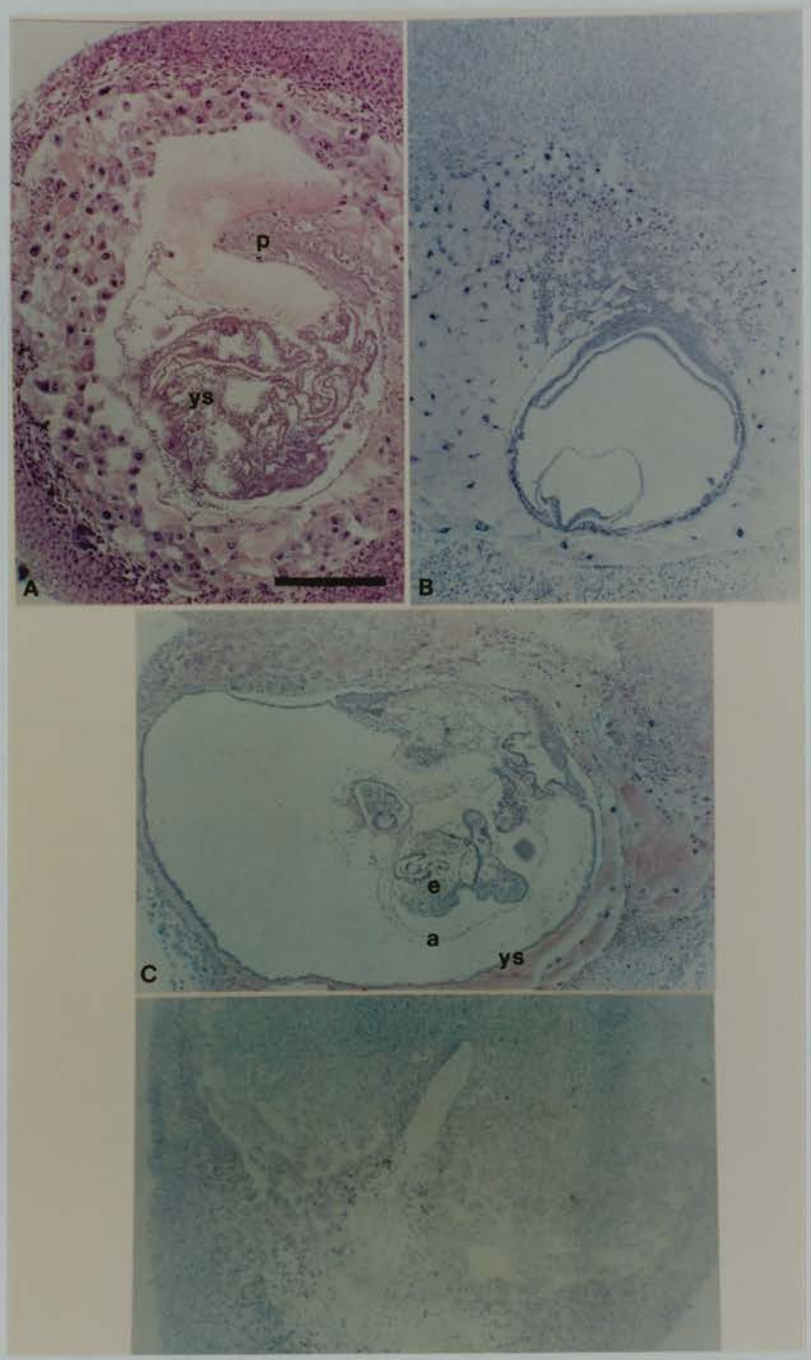


Fig 4.2A. 10.5 day CBA/J x DBA/2 conceptus with a small amount of placental tissue and a disorganized yolk sac.

Fig 4.2B. A retarded 10.5 day CBA/J x DBA/2 conceptus, similar in appearance to an 8.0 - 8.5 day embryo.

Fig 4.2C. A resorbing 10.5 day CBA/J x BALB/c conceptus with no identifiable embryonic structures.

Fig 4.2D. The necrotic remains of a CBA/J x CBA/J, 10.5 day conceptus.
Scale bar = 500μm.
a = amnion, e = embryo, p = placenta, ys = yolk sac.

one was similar to the (CBA/J x DBA/2) conceptus shown in Fig 4.3A (Fig 4.3B), with the extraembryonic membranes surviving and the embryo appearing necrotic and dying having been attacked by infiltrating cells.

At 12.5d in the experimental (CBA/J x DBA/2) cross, again 6 of the embryos appeared grossly abnormal. The decidual swellings contained cellular remains of the dying embryos. One embryo was similar in appearance to Fig 4.3A, with Reichert's membrane, the yolk sac and amnion remaining, but the 'embryo' had degenerated further (Fig 4.4). The remaining embryos were moles with fragments of embryonic material surviving. In the (CBA/J x BALB/c) control, 2 were moles and one was similar to the embryo shown in Fig 4.4A with a small amount of embryonic material remaining. At this stage in the (CBA/J x CBA/J) cross, the 3 abnormal embryos were moles, two had remnants of embryonic material and one did not.

Table 4.4 shows the varying types of abnormality that were detected in the histological study and the number of embryos from each cross with this morphology. The 5 different types of abnormality are given as tilted, when the 9.5d.p.c. embryos were observed to be retarded and 'tilted' within the decidua, resorbing fetus, where the fetus looks necrotic and dying within the decidual swelling, empty sac where the extraembryonic membranes are present but there is no embryo, mole with some embryonic remains, where the embryo has been resorbed but some recognizable tissues remain and mole with no recognizable embryonic remains, where the embryos has been more completely resorbed. In the experimental (CBA/J x DBA/2) cross the majority of the abnormal conceptuses examined were those that had a necrotic fetus within the decidual swelling, the next most common abnormality was the tilted embryos but these were only observed at 9.5d.p.c. and were all observed in the samelitter. In the CBA/J x BALB/c allogeneic control cross, only 6 abnormal conceptuses were examined and these were either moles (with and without embryonic remains) or the empty sac structure. In the (CBA/J x CBA/J) cross, which

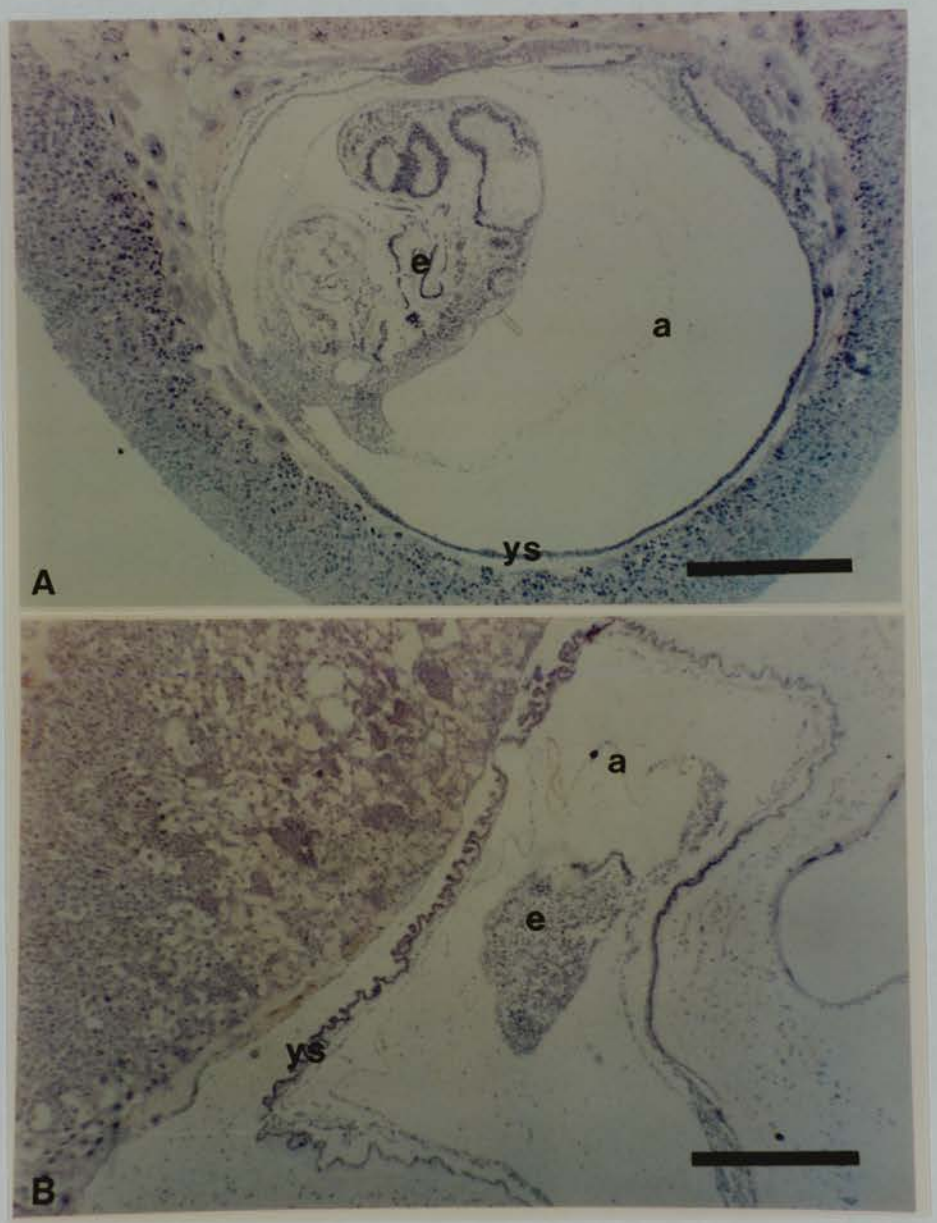


Fig 4.3 A. Necrotic remains of resorbing 11.5 day CBA/J x DBA/2 conceptus.
Fig 4.3 B. Resorbing 11.5 day CBA/J x CBA/J conceptus.
Scale bar = 500μm.
a = amnion, e = embryo, ys = yolk sac

Table 4.4 Summary of the histological observations of all the embryos examined at various ages.

Age (d.p.c.)	Normal embryos	Tilted embryo	Resorbing fetus	Empty sac	Mole[a]	Mole[b]
CBA/J x DBA/2						
9.5	22	5	1	0	0	0
10.5*	20	0	0	1	0	0
11.5	21	0	6	0	0	0
12.5	21	0	1	0	4	1
Total	84	5	8	1	4	1
CBA/J x BALB/C						
9.5	6	0	0	2	0	0
10.5	14	0	0	0	0	1
11.5	20	0	0	0	0	0
12.5	15	0	0	0	1	2
Total	55	0	0	2	1	3
(CBA/J x CBA/J)						
9.5	5	1	0	1	0	2
10.5	11	0	1	0	0	5
11.5	13	0	1	0	0	4
12.5	16	0	0	0	2	1
Total	45	1	2	1	2	12

[a] Mole with some embryonic remains

[b] mole with no recognizable embryonic remains

* One conceptus at this stage was retarded by approximately 2 days.

had the highest proportion of abnormal embryos, moles with no recognizable embryonic structures were predominant. However, there were conceptuses in this group from each of the 5 types described.

IN SITU HYBRIDISATION

The *in situ* hybridisation experiment was set up to compare the pattern of expression of TNF-a positive cells at the fetal-maternal interface (the boundary between the fetal and maternal tissue) in 12.5d.p.c. (CBA/J x DBA/2) normal and resorbing embryos, CBA/J x BALB/c control embryos and abnormal and normal conceptuses from *a/m* females mated to *b/m* males (same cross as in section 2.2). Observed and expected results from a typical *in situ* experiment are shown in Table 4.5. The expected results were compiled with reference to previously published data (Clark *et al*, 1990, Lea *et al*, 1992). Some TNF-α positive cells would be expected in the 12.5d.p.c. normal (CBA/J x BALB/c) and normal (CBA/J x DBA/2) embryos. The resorbing (CBA/J x DBA/2) embryos would be expected to have a greater number of TNF-α positive cells more heavily labelled with probe (Lea *et al,* 1992) compared with the normal embryos. The same proportion of TNF-α positive cells expected in the normal CBA/J x DBA/2 embryos would be expected in the normal BGO embryos. The predictions for the expression of TNF-α in the resorbing BGO embryos, could again be similar to the normal CBA/J x DBA/2 embryos, but, if TNF-α positive cells are involved in the resorption of these embryos, then a greater number of heavily labelled TNF-α positive cells would be expected. In the 10.5 day BGO embryos, TNF- α positive cells may be detected in the normal and resorbing embryos, in the case of the resorbing embryos, the TNF- α positive cells may be more abundant with a higher degree of labelling. TNF- α positive cells were observed in the trophoblast giant cell region of the 12.5 day CBA/J x BALB/c control embryo, also in the CBA/J x DBA/2 resorb and normal embryo and the 12.5 day BGO normal and resorbing embryos. However, a high degree of non-specific

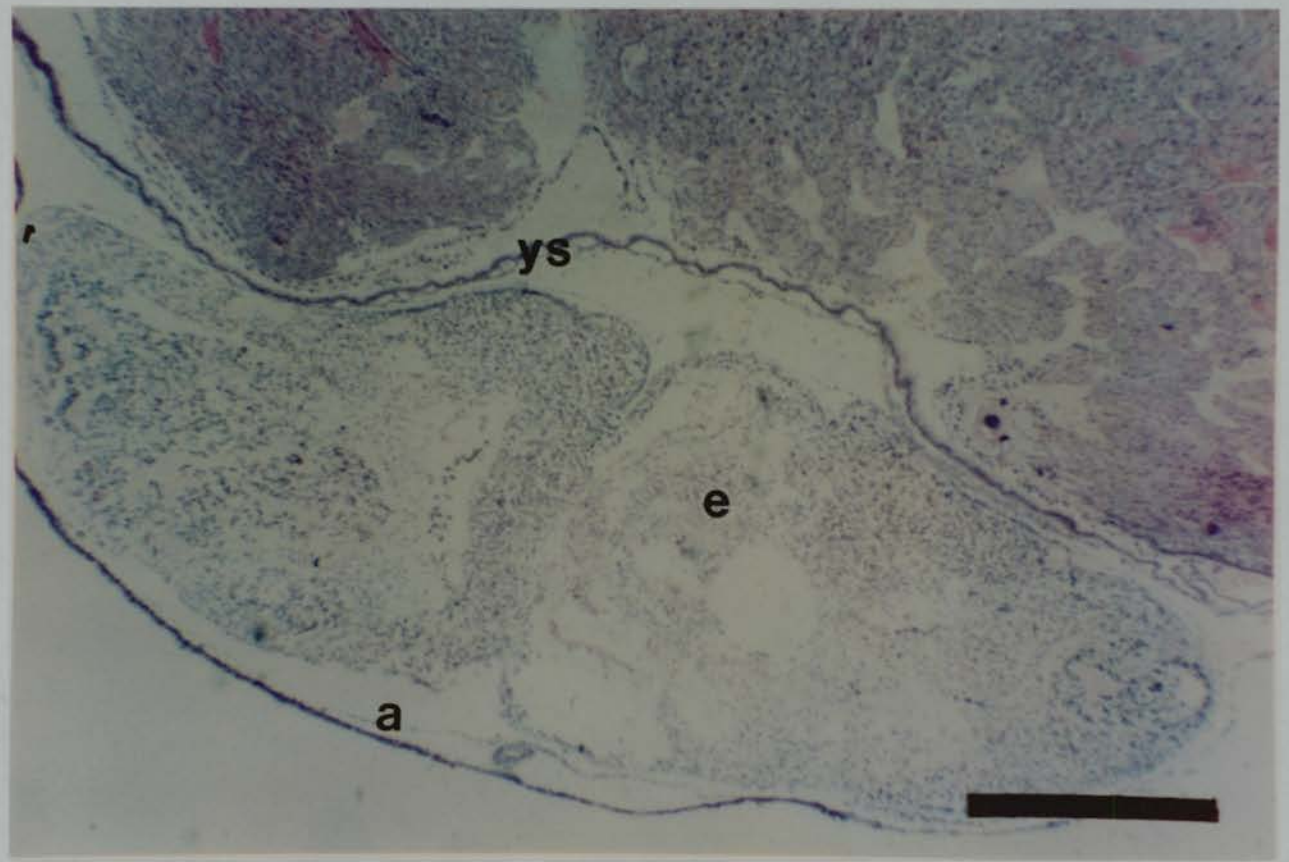


Fig 4.4. A resorbing 12.5 day CBA/J x DBA/2 conceptus. Scale bar = 500 µm.
a = amnion, e = embryo, ys = yolk sac.

hybridisation was also apparent in these implants. No specific TNF- α staining was apparent in the 10.5 day BGO normal embryo but the resorbing embryo had a few very dense clusters present.

A high degree of non-specific binding of the negative control 1kb ladder probe was observed in these studies (Table 4.5). The non-specific clusters clearly had a random distribution over the implant, however this did include the feto-maternal interface, where positive hybridisation to TNF- α has previously been observed (Clark et al, 1990, Lea *et al*, 1992). Further optimisation of the *in situ* technique should be undertaken before any conclusions can be drawn from this preliminary study.

4.4 DISCUSSION

a) Frequencies of abnormal and resorbing embryos

Chaout, 1989, reported that the resorption rate observed in the (CBA/J x DBA/2) cross varied from one breeding centre to another and it was thought that infection played a role in the resorptions observed in this system (Hamilton and Hamilton, 1987). More recently Baines *et al* (1994) have shown that resorption rates are similar in specific pathogen free housed CBA/J female mice mated to DBA/2 males and CBA/J female mice infected with viral pathogens and housed in the same conditions. Also treatment of the CBA/J females with the antiviral drug, ribavirin (1-b-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) increased rate of resorption, caused retardation in embryonic development and also impaired development of the trophoblast (Clark *et al,* 1993). This does not rule out the involvement of all pathogens (e.g. mycoplasma). The results from the morphological and histological study presented here show that resorption rate also varies within the same centre.

The starting point for the morphology study was taken as 11.5d.p.c., when the highest increase in frequency of resorptions was reported in the Baines *et al* study, this was also extended to include 12.5 and 13.5 day old embryos. However, only

Table 4.5 Results from a typical TNF-α*in situ* hybridisation run.

Mating	Age	Embryo	Observed				Expected			
			TNF-α		1kb L		TNF-α		1kb L	
			T	D	T	D	T	D	T	D
CBA/J x BALB/c	12.5	Normal	++	-	+	+	+	+	-	-
CBA/J x DBA	12.5	Normal	+	-	++	+	+	+	-	-
		Abnormal	+	+	+	+	++	++	-	-
BGO (*a/m* x *b/m*)	12.5	Normal	+	-	+	-	+	+	-	-
		Abnormal	+	+	+	+	≥+	≥+	-	-
BGO (*a/m* x *b/m*)	10.5	Normal	-	+	+	+	±	±	-	-
		Abnormal	++	+	++	+	≥±	≥±	-	-

T = trophoblast, feto-maternal interface.
D = decidua
1KbL= 1Kb ladder control probe see Appendix III(b)

Table 4.6 Comparison of the resorption frequencies in CBA/J x CBA/J matings.

	Total no. embryos	Normal	Resorb	% Resorption
Leonard et al (1971)	-	-	-	12.33
Hamilton & Hamilton (1987)	35	31	4	11.4
Hamilton & Hamilton (1987)	27	23	4	14.8
Baines *et al* (1994)	(72)	-	-	5.2±7.3*
Present study	63	45	18	28.6

* ± standard deviation
Number in brackets was calculated from the the number of females x mean number of implantations per female from Table 4.1B.

5.6% of the embryos from the experimental cross examined were resorbing at this stage, and at 12.5 days, the number of resorption has only increased to 8.2%. At 13.5d.p.c. the frequency of resorptions was 5.1%. These are rather low frequencies when other investigators have observed a resorption frequency of approximately 30% in this cross (Clark, 1980, Chaout, 1989, and Baines *et al*, 1994) and in normal mouse pregnancies approximately 10% of embryos die in utero (Leonard *et al* 1971). The frequency of resorptions in the control CBA/J x BALB/c cross in the present study, were similar at 12.5.and 13.5d.p.c if not slightly elevated compared to the experimental data. In the histological study a higher incidence of abnormal/resorptions were observed in the CBA/J x DBA/2 cross (19.2%) and this was comparable with the published data of Baines et al, (1994) shown in Table 4.1. This was higher than the (CBA/J x BALB/c) controls but this difference was not statistically significant. However, one difference between the present study and that of Baines *et al*, (1994) was the greater frequency of abnormal or resorbing CBA/J x CBA/J embryos in my study (see Table 4.3 and 4.1B). One possible reason for the difference observed in the resorption frequencies between these two studies could be that a different batch of male DBA/2 mice were used for the morphology study.

In the histological study, conceptuses were examined at 9.5 days, 2 days before the highest incidence of resorption was reported in the Baines *et al* study, through to 12.5d.p.c. We hoped to be able to record changes that were occuring within the resorbing conceptuses of the (CBA/J x DBA/2) pregnancies before signs of resorption were seen grossly. At 9.5 days a cellular infiltrate and the presence of what appeared to be dead trophoblast giant cells was associated with all of the abnormal conceptuses examined. The abnormality apparent at this stage in the experimental cross, was that the embryos were tilted within the decidua and retarded by approximately 2 days. As all of these embryos were littermates it is unclear whether these features are characteristic of 'pre - resorbing' (CBA/J x DBA/2) conceptuses or whether they are due to an unrelated problem associated with the

pregnancy. A similar looking embryo was also found in the (CBA/J x CBA/J) cross at this stage. The majority of the embryos examined at this age were retarded, but were otherwise normal.

In the later age groups of the (CBA/J x DBA/2) embryos examined, the majority of the abnormal embryos were resorbing fetuses or moles (Table 4.4). In the (CBA/J x BALB/c) and (CBA/J x CBA/J) groups, the resorptions were most commonly moles. In some, a small amount of embryonic material remained, but in the majority an amorphous mass was present with no distinguishable embryonic material. Because of the presence of a small amount of embryonic material detected within some of the moles, it could be assumed that embryo formation and subsequent death had occurred. In the cases where no embryonic derivatives were detected complete resorption had taken place. An infiltrate of cells from the decidua was often associated with the resorption and based on the findings of Gendron *et al* (1988), these are most likely to be polymorphonuclear leukocytes at the stages examined. Immunohistochemical studies are necessary to determine the identity of these cells.

An elevated number of abnormal and resorbing conceptuses were observed in the syngeneic (CBA/J x CBA/J) pregnancies. The frequency of resorptions observed in this cross was not significantly higher than the CBA/J x DBA/2 cross but was higher than the control CBA/J x BALB/c cross. Previous studies looking at the resorption frequencies of (CBA/J x CBA/J) matings have not observed this high proportion of resorptions (Table 4.6) and its cause remains unexplained.

b) TNF-α expression

The immunological mechanisms that cause the resorptions in the DBA/2 mated CBA/J pregnancies have been partially elucidated. In vitro studies have shown that the release of a TGF-β_2 factor from cultured decidual tissue is delayed (10.5d.p.c) with respect to TNF- α (8.5d.p.c.). In normal pregnancies these antagonistic factors

are released simultaneously (Clark *et al.*, 1991). It has been postulated that in the absence of TGF-β_2 , TNF- α activates NK cells into lymphokine activated killer cells (LAKs), which are able to destroy trophoblast. It should be noted that trophoblast is resistant to other immunocompetent cell types (NK, NC and cytotoxic T lymphocytes). Other abortogenic cytokines (IFNγ and IL-2) have been found at elevated levels in the placentae of (CBA/J x DBA/2) conceptuses (Tangri & Raghupathy, 1993) and it is thought that these also have a role in the cascade of events that result in the resorption of these conceptuses.

Morphologically the later stage resorbing embryos appear similar in all the age groups examined in this study. The resorption process likely involves a common cascade of local cellular and immunological events, however the trigger for resorption in the crosses assessed may be different. In the present study, it has not been determined if the cytokine profiles in the early stages of resorption differ between pregnancies, i.e. (CBA/J x DBA/2), (CBA/J x BALB/c), the (CBA/J x CBA/J) or the homozygous GPI null cross. A further improvement on the present study would be to perform immunocytochemistry with antibodies to TNF- α or TGF-β_2 in conjunction with histology to identify which of the embryos were resorbing due to the postulated immunological problems associated with the (CBA/J x DBA/2) system. Without this it is not completely clear whether the resorbing embryos observed are dying of the same or different causes. This idea does not appear to have been fully addressed in many of the studies looking at the immunological causes of resorptions. Individual implant sites have been shown to have different levels of TGF-β_2 mediated suppressor activity (Clark *et al* 1980). Tanghri and Raghupathy (1993) also showed that placentae of DBA/2 mated CBA/J mice had elevated levels of TNF- α and other cytokines potentially detrimental to pregnancy. However, it is never stated clearly whether any resorptions are found in the control crosses used in these studies, approximately 10% is the normal value quoted for spontaneous resorption (Leonard *et al* 1971), although this can vary

between strains, and the present study has shown that this value can be even higher. It would be interesting to study the cytokine profiles of these sporadically resorbing conceptuses in the same way as the resorbing (CBA/J x DBA/2) conceptuses to identify whether similar mechanisms are involved.

For any information to be gained from the *in situ* hybridisation study, the protocol would have to be further optimised for the tissue being examined. Lower temperatures of hybridisation could be attempted to try to lower the non-specific binding of the negative control probe, or perhaps more stringent washes should be attempted to remove excess probe after hybridization. Another way to try to remove excess probe would be to use a rocking table while washing the slides after hybridisation. As the preservation of the fetal material on the slides was not good, the proteinase K digestion step may have been too severe for this tissue. Although a range of proteinase K concentrations were tested as well as a milder pronase digestion, the selected proteinase K concentration may not have been optimal. Furthermore, the time of the digestion step could be altered in order to reduce this problem. It is well established that the optimisation of an *in situ* hybridisation method for a specific probe and tissue requires a great deal of technical time which unfortunately was limited in this part of the project.

c) Comparison of the two models of embryonic loss

The resorbing conceptuses produced in the (CBA/J x DBA/2) cross differ from the homozygous GPI null embryos because they occur later in development. The homozygous GPI null embryos fail to develop beyond gastrulation, whereas in the (CBA/J x DBA/2) conceptuses abnormalities do not become apparent until a later stage, approximately 9.5d.p.c. The fetus appears to be resorbed before the extraembryonic membranes, in some cases when a necrotic fetus was observed the extraembryonic membranes were still recognizable. However, in the various moles observed when degeneration of the fetus was almost complete, the extraembryonic

membranes were not distinct structures. So, although the fetus is resorbed before the membranes an empty sac structure similar to that of the homozygous null embryos was not apparent.

Because resorption of the homozygous GPI null embryos occurs earlier than the (CBA/J x DBA/2) resorptions, (the homozygous GPI null embryos are completely resorbed by approximately 11.5d.p.c. and the (CBA/J x DBA/2) resorbing embryos still have some embryonic material present by 12.5d.p.c.), TNF- α positive cells may not be present at the same time in the different resorptions. Once implantation has occurred, the trophoblast represents the main immunologic challenge to the maternal immune system. This may be attacked in the CBA/J x DBA/2 model and lead to resorption. In the homozygous GPI null model, the embryo dies and leaves an apparently intact wall of trophoblast until 9.5d.p.c. Despite the degeneration of the homozygous GPI null embryo, an immunological response may not occur until the trophoblast has degenerated, perhaps due to inadequate stimulation of decidual TGF β_2 release. The final steps leading to the resorption are likely the same, but the initiation and timing are likely different.

PART II

THE DEVELOPMENTAL POTENTIAL OF HOMOZYGOUS GPI NULL CELLS

CHAPTER 5

CAN HOMOZYGOUS GPI NULL CELLS BE RESCUED IN FETAL AGGREGATION CHIMAERAS?

5.1 INTRODUCTION

The histological study (Chapter 2) showed that homozygous GPI null embryos (*Gpi-1s*$^{m/m}$) can survive until 7.5 days and that cells can survive until 10.5 days as disorganized tissues. Could homozygous GPI null cells could be rescued and survive for longer in different tissues when combined with normal cells in aggregation chimaeras? As shown in Chapter 1, chimaeras are useful tools for studying development. They can be used to trace the lineage of marked cells, to estimate the number of founder cells of a particular organ and to test the developmental potential of different cells. In this study, chimaeras were employed to examine the developmental potential of homozygous GPI null cells when combined with normal cells.

Chimaeras were produced by aggregating two 8-cell embryos and analysing their development at 12.5 d.p.c. Because the homozygous genotype is embryo-lethal, two heterozygotes carrying a null allele of *Gpi-1s* were intercrossed, only 25% of the chimaeras produced would be expected to contain homozygous cells (if they survived at all). Ideally, *Gpi-1s*$^{a/m}$ mice should be crossed to *Gpi-1s*$^{b/m}$ mice so that each of the four progeny genotypes can be identified (i.e. *a/m*, *b/m*, *a/b* and *m/m*) after electrophoresis. Initially, while waiting for the correct stocks of mice to be produced, experiment (i) was performed. Once the other stock became available, series (ii) was carried out.

The markers used in this study were GPI, the two components of the chimaera varying at the *Gpi-1s* locus, and eye pigmentation, one component of the chimaera being pigmented, the other albino (hybrid strain CF1). A reiterated transgenic marker was also included, as discussed in Chapter 6.

5.2 MATERIAL AND METHODS

CHIMAERA PRODUCTION

Three different series of chimaeras were produced, two experimental series (i & ii) and a control series (iii). GN and CF1 females (see Appendix I) were superovulated (see Appendix VII) and mated to NUL and CF1 males respectively. In the following description italicised letters represent different alleles of the *Gpi-1s* locus (*a,b,c,m*) on chromosome 7.

(i) [GN (*b/m*) x NUL (*b/m*)]↔ [CF1(*c/c*) x CF1 (*c/c*)] to produce chimaeras of GPI genotypes *b/m*↔*c/c*, *b/b*↔*c/c* and *m/m*↔*c/c*

(ii) [GN (*b/m*) x NUL (*a/m*)] ↔ [CF1(*c/c*) x CF1 (*c/c*)] to produce chimaeras of the GPI genotypes *a/m*↔*c/c*, *a/b*↔*c/c* , *b/m*↔*c/c* and *m/m*↔*c/c*

(iii) [BF1 (*b/b*) x NUL (*b/m*)] ↔ [CF1(*c/c*) x CF1 (*c/c*)] to produce chimaeras of the GPI genotypes , *b/b*↔*c/c* , *b/m*↔*c/c*

The NUL males were also hemizygous for a β globin transgenic marker (see Chapter 6 and Appendix III(c)).

Embryos were flushed from the oviduct and uterus at $2^1/_2$ days *post coitum* (p.c.), at the 8-cell stage After collection, the zonae pellucida were removed in acid Tyrode's solution at pH 2.5 and the denuded embryos were aggregated into pairs (see Appendix VI). Aggregated pairs consisting of one (GN x NUL) embryo and one

(CF1 x CF1) embryo were cultured overnight at 37°C in 5% CO_2 in air and transferred the next day to CF1 pseudopregnant females.

EMBRYO TRANSFER

CF1 pseudopregnant females were used as recipients for all of the chimaera experiments so that any maternal contamination would result in an elevated proportion of GPI-1C in tissues which were contaminated and therefore would not interfere with identification of putative null chimaeras, which would produce only GPI -1C enzyme.

EMBRYO DISSECTIONS

For all chimaeras, the recipient females were sacrificed at $12^1/_2$ days p.c., and the resulting conceptuses were analysed in the same manner. The weights of the total conceptus were recorded. A small piece of the tail and yolk sac were removed for GPI analysis. For conceptuses Sx41 - Sx59 in series (i) and all of those in series (ii) and (iii), half of the placenta was also included. These samples were stored at -20°C in a multiwell plate containing either 10µl (tail and yolk sac) or 50µl (placenta) of 50% glycerol in water. A rough estimate of the contribution of the GN x NUL (pigmented) component in the chimaeras was made by observing the distribution of pigmented cells in the retinal epithelium of the eye at the time of dissection. After removal of samples for GPI analysis, the conceptus was fixed and processed for histology and if appropriate *in situ* hybridisation to a reiterated β-globin transgene. A second estimate of the % eye pigment was made from histological sections of some of the chimaeras (see below).

GEL ELECTROPHORESIS

The method of enzyme electrophoresis used in this study has been described previously (West and Green, 1983; West et al, 1986). Electrophoresis was carried

out using electrophoresis tanks and Super Z-12 applicators obtained from Helena Laboratories and a Consort Bioblock Scientific power pack. Titan III cellulose acetate plates (Helena Laboratories) were soaked in electrophoresis buffer (see Appendix IV) for at least 30 min The tank reservoirs were filled with electrophoresis buffer and Whatman filter paper was used to form wicks. Samples were applied to blotted plates and run from anode to cathode at 200V for 60min, then stained (see Appendix VI) for up to 20min on a 37°C hotplate. After rinsing in water to remove excess stain, the plates were fixed in 5% acetic acid for 5min then washed in distilled water for 15min. The plates were then air dried in the dark. Scanning densitometry was then performed to determine the relative proportions of GPI-1A, GPI-1B and GPI-1C in the tissue samples.

ASSESSMENT OF SENSITIVITY OF ASSAY SYSTEM

The sensitivity of the staining technique was tested using samples of known proportions of GPI-1B and GPI-1C fetal homogenates. This was achieved by mixing mouse embryo homogenates from a mouse strain homozygous for GPI-1B with that of a strain homozygous for GPI-1C. Proportions ranging from 100% B tissue:0% C, through 50%:50% to 0% B:100% C were used. The bands were separated by gel electrophoresis and scanned to compare the known percentage of tissue in the sample mixture with the percentage of each GPI type detected on the gel. Three repeats using independant mixtures of homogenates were run. A graph was plotted of the expected percentage of GPI-1B in the fetal homogenate sample against the mean percentage of GPI-1B detected in the samples by the scanner. Lines have also been plotted to show what the expected % GPI-1B would be if the activity of GPI-1C was 50%, 75% and 100% of GPI-1B. The plot lies very near to the values expected if the specific activity of GPI-1C was 75% of the activity of wild type GPI-1B enzyme (Fig 5.1). Previous studies have shown that the GPI-1C allozyme is unstable which would explain why it has a lower specific activity than GPI-1B (West et al.,1987). A

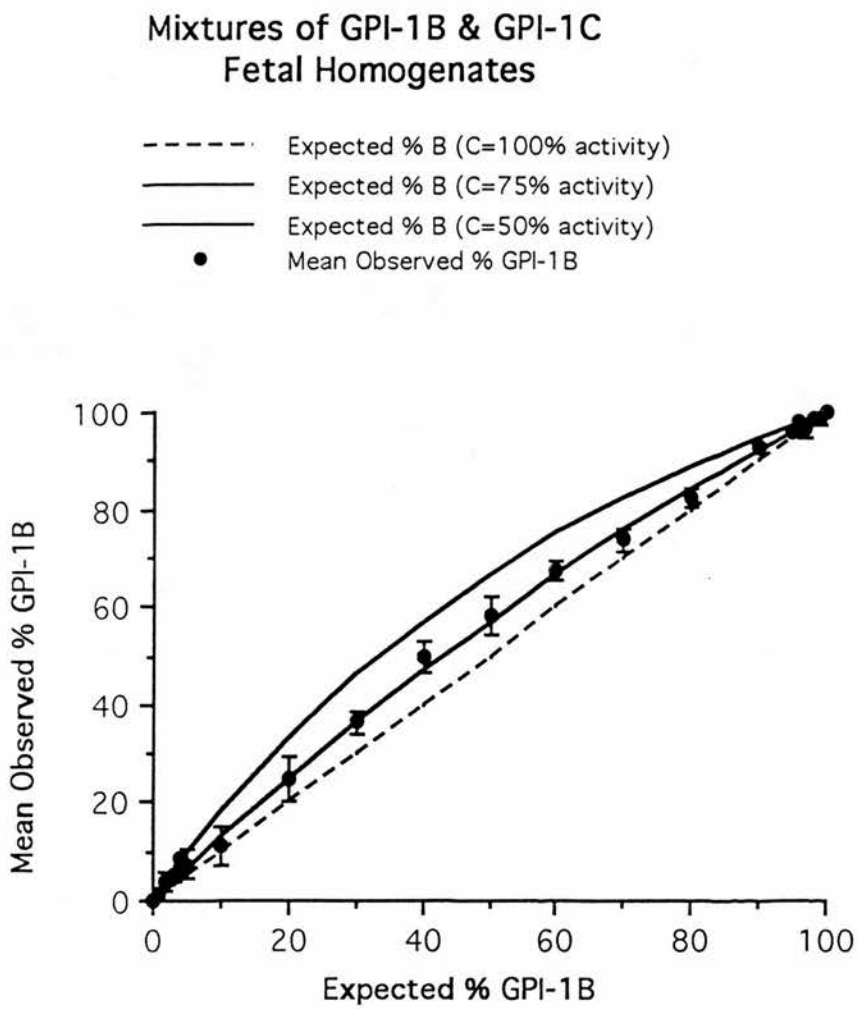


Fig 5.1 Graph showing the mean observed % GPI-1B detected in mixtures of GPI-1B and GPI-1C fetal homogenates plotted against the expected GPI-1B. Also shown are the plots that would have been expected if the activity of GPI-1C was 50%, 75% and 100% the activity of GPI-1B.

further 3 repeat runs were carried out to test the limit of detection of GPI 1 B in the homogenate samples by the scanner. The dilutions were increased in 1% intervals, from 0 to 5%, then 10 and 20%. The results are shown in Table 5.1. It can be seen from this that 3% of GPI-1B present in the homogenate could always be detected by the scanner. Below this value, the scanner could not reliably detect the presence of a small percentage of GPI-1B mixed with GPI-1C.

The formulas used to calculate the expected % GPI-1B if GPI-1C was 50% and 75% the activity are:

(a) Expected %GPI-1B(in B + C mixtures) if C=50% wild type activity

$$= \frac{\text{Observed \% GPI-1B} \times 100}{\text{Observed \% GPI-1B} + (\text{Observed \% GPI-1C} \times 100 / 50)}$$

(b) Expected % GPI-1B(in B + C mixtures) if C= 75% activity of wild type

$$= \frac{\text{Observed \% GPI-1B} \times 100}{\text{Observed \% GPI-1B} + (\text{Observed \% GPI-1C} \times 100/75)}$$

HISTOLOGY

After dissection of the tissues for electrophoresis, the remaining part of the embryos were fixed in 3:1 ethanol:acetic acid overnight. The samples were then passed through a graded series of ethanol, followed by HistoClear (National Diagnostic) in order to dehydrate the samples, and then immersion in 50:50 Histo-Clear:paraffin wax mixture, before paraffin wax under vacuum. After processing, the samples were embedded in wax and stored at 4°C Sections of the embryos were cut at 7μm .

Table 5.1 showing the observed GPI-1 allozymes detected in known dilutions of 3 series of mixtures of GPI-1B + GPI-1C fetal homogenates

Expected % GPI-1B by tissue weight in homogenate	Observed GPI-1 allozymes		
	Replicate 1	Replicate 2	Replicate 3
0	C	C	C
1	B + C	C	C
2	B + C	B + C	C
3	B + C	B + C	B + C
4	B + C	B + C	B + C
5	B + C	B + C	B + C
10	B + C	B + C	B + C
20	B + C	B + C	B + C

EYE PIGMENT ANALYSIS

Eye pigment analysis was performed in collaboration with Mr. B. A. Hodson, Department of Obstetrics and Gynaecology, University of Edinburgh (see Chapter 7).

Rough estimates of the percentage of eye pigment were made upon dissection. Once the chimaeras were sectioned a second estimate was made from histological sections, as described by West (1976). Serial sections of the eye were cut at 7μm and stained with haematoxylin and eosin (Fig 5.2). A mid section of the eye was used for analysis. Analysis was carried out using an Olympus BH-2 microscope with a Sony CCD/RGB camera model DXC-151P attached. The image analysis program Colour Vision 1.6.3 (Improvision) was run on a Macintosh Quadra 700. Analysis always started at the choroid fissure. The length of pigmented and unpigmented patches were recorded around the circumference of the eye. From this the percentage of pigment in the midsection of each eye was calculated.

5.3 RESULTS

Approximately 25% of (GN x NUL) embryos should be homozygous for the null allele of *Gpi-1s*. All (CF1 x CF1) embryos are albino and homozygous for *Gpi-1s*^{c}. The different GPI genotypes that will be produced when chimaeras are made by aggregating embryos from these crosses are shown in Table 5.2. In each case, one genotypic component of the chimaera can be distinguished from the other by GPI electrophoresis. A fetus would be considered to be chimaeric if both of the genotypes contributing to the chimaera, determined by pigment variegation and/or GPI electrophoresis, are present in the fetus. For a chimaera to be classified as a homozygous null chimaera, GPI-1C only must be present in the tissues analysed and eye pigmentation observed (the GPI-1C allozyme is produced by the albino component of the chimaera).

Table 5.2 The expected chimaera types that would be produced in series (i), (ii) and (iii)

Genotype of chimaera	Phenotype Pigment	GPI
Series (i) (N = 59)		
GN (*b/m*) x NUL (*b/m*) ↔ CF2 (*c/c*)*		
b/m ↔ *c/c*	variegated	B + C
b/b ↔ *c/c*	variegated	B + C
m/m ↔ *c/c*	variegated	C
non chimaeric c/c	albino	C
non-chimaeric *b/m*, *b/b*, or *m/m*	pigmented	B
Series (ii) (N = 52)		
GN (*b/m*) x NUL (*a/m*)↔CF2 (*c/c*)		
b/m↔ *c/c*	variegated	B + C
a/m ↔ *c/c*	variegated	A + C
a/b ↔ *c/c*	variegated	AB + C
m/m ↔ *c/c*	variegated	C
non chimaeric c/c	albino	C
non chimaeric *b/m*, *a/m*, *a/b*, *m/m*	pigmented	B, A, or AB
Series (iii) (N = 24)		
BF1 (*b/b*)x NUL (*b/m*) ↔ CF2 (*c/c*)		
b/m ↔ *c/c*	variegated	B + C
b/b ↔ *c/c*	variegated	B + C
b/m ↔ *c/c*	albino	B + C
b/b ↔ *c/c*	albino	B + C
non chimaeric *c/c*	albino	C
non chimaeric *b/m* or *b/b*	pigmented	B

* CF2 (*c/c*) = CF1 (*c/c*) x CF1 (*c/c*)

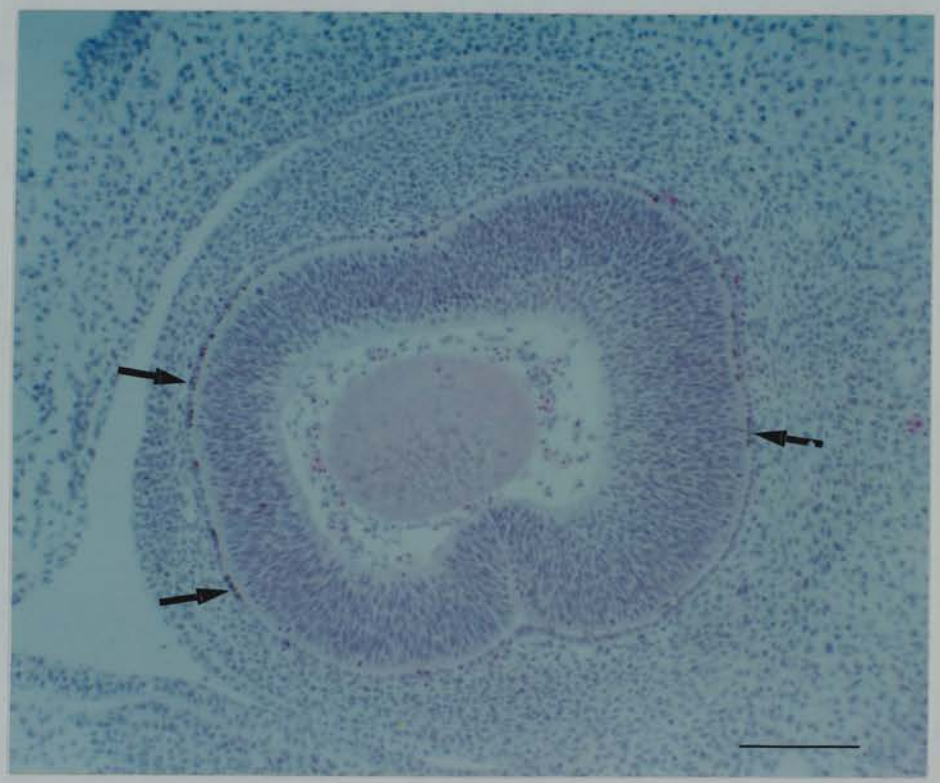


Fig 5.2 Mid-section of 12.5 day chimaeric eye. Arrows indicate pigment.

In the first chimaera group 59 chimaeras were produced, in the second group 52 were produced and in the control series, 24 were recovered.

A subjective estimate of eye pigmentation was made upon dissection (Est. 1, Tables 5.3 - 5.7) but this was often difficult because the fetal membranes were not removed. A more objective estimate was made from histological sections (Est. 2, Table 5.3 - 5.7). One eye was analysed from all the fetuses from experimental series (ii), the control series (iii) and in 20/59 fetuses in series (i). These included the 14 fetuses that produced only GPI-1C and 6 others (GPI-1B + GPI-1C). These six were chosen because of the low proportion of pigment observed at the time of dissection (Est 1). Initial eye pigmentation analysis at the time of dissection (Est 1) identified 38 chimaeras in series (i), 33 in series (ii) and 20 chimaeras in series (iii) to be at least partly pigmented. The initial estimate was very crude because the fetal eye was often obscured by membranes, which were left in situ for the transgenic analysis (Chapter 6). This may account for some of the discrepancies between the values for Estimate 1 and Estimate 2 in Tables 5.3 - 5.7. The subsequent histological examination of a single mid-section of the eye probably gave a more reliable estimate of the true % pigment in the fetal eyes (Est 2). A previous study showed that estimation of % pigment from a single section correlated very closely with the chimaeric composition in the fetal trunk, estimated by GPI electrophoresis (Hodson, Keighren and West, unpublished). After this analysis, 36 chimaeras were at least partly pigmented in series (ii) and 20 in series (iii). In series (i) 5 of the 14 GPI-1C only fetuses had variegated eyes and the other 9 had albino eyes. Three of the 6 GPI-1B + C chimaeras tested in series (i) had variegated eyes and 3 had albino eyes. Histological sections were not prepared from the remaining 39 fetuses in series (i).

GPI electrophoresis was performed to try to distinguish which of the fetuses with pigmented or variegated eyes were putative homozygous null chimaeras (*m/m* $\leftrightarrow$ *c/c*). Electrophoresis was performed on a sample of tail and yolk sac from each of

the first 40 chimaeras produced in series (i). After this and in subsequent studies (series (ii) and (iii)) a piece of placenta was also taken. Each sample was run at least twice to confirm the result. Fig 5.3 shows a typical GPI electrophoresis plate showing the bands that would be expected from chimaeric offspring in series (ii).

Of the 59 conceptuses in series (i), 18 fetuses had only one GPI type present (4 were GPI-1B and 14 were GPI-1C only) and the remaining 41 were chimaeric (GPI allozymes from each component of the chimaera were present in at least one of the tissue samples taken, Tables 5.3 and 5.4. Twelve out of the 41 fetuses detected as chimaeric by GPI electrophoresis had no detectable eye pigmentation. The remaining 29 in this series had varying amounts of eye pigmentation present (Table 5.3). Twelve conceptuses were recovered that had albino eyes (by either Est 1 or Est 2) but were GPI-1B and GPI-1-C (Group (a) Table 5.3) and 29 conceptuses were observed to have variegated eyes (by either Est 1 or Est 2) and produced both GPI-1B and GPI-1C (group (b) Table 5.3). Two fetuses (Group (c) in Table 5.3) produced only GPI-1B, but had variegated eyes. These could be chimaeric but, since no histology was done, the apparently incomplete pigmentation could have reflected an immature fetus with dilute eye pigment rather than true variegation. Two other fetuses produced GPI-1B only but were estimated as being 100% pigmented, these were probably non-chimaeric (*b/b* or *b/m*) fetuses but they could be chimaeric fetuses where the proportion of GPI-1C cells present in the tissues analysed was very much smaller than GPI-1B (Group (d) in Table 5.3), and was below the limit of detection of the assay system.

Nine of the fetuses that were GPI-1C only in the tissues examined were albino in the eye (Group (e), Table 5.4). These were probably non-chimaeric (*c/c*) fetuses but could have included some chimaeras in which the *c/c* component was overwhelmingly predominant in the tissues analysed. The 5 remaining fetuses that were GPI-1C only had some eye pigment and therefore were genuine chimaeras

Fig 5.3 Electrophoresis plate showing expected bands from chimaeric offspring in series (ii). Lane 1 GPI-1A + C, Lane 2 GPI-1B + C, Lane 3 GPI-1AB + C, Lane 4 GPI-1C and Lane 5 GPI-1AB.

Table 5.3 Results from series (i) (*b/m* x *b/m*) ↔ *c/c* , conceptuses. Conceptuses with GPI-1B and GPI-1C or only GPI-1B (therefore not *m/m* ↔ *c/c* chimaeras). Ranked by % GPI-1B present in tail.

Chimaera no.	Weight of conceptus (mg)	% Pigment (Est. 1)	% Pigment (Est. 2)	%GPI-1B		
				Tail	YS	Placenta
(a) Conceptuses with albino eyes but both GPI-1B and GPI-1C: *b/b*↔ *c/c* or *b/m* ↔ *c/c*						
Sx37	330.3	0	0	9.2	0	N.D
Sx21	291.3	0	N.D	9.9	17.4	N.D
Sx7	300.3	0	N.D	10.1	25.3	N.D
Sx54	339.0	2	0	10.3	27.4	0
Sx14	350.8	1	0	12.7	0	N.D
Sx8	319.1	0	N.D	14.3	87.7	N.D
Sx20	314.7	0	N.D	16.2	6.2	N.D
Sx3	298.2	0	N.D	17.8	0	N.D
Sx17	286.1	0	N.D	23.9	0	N.D
Sx27	360.6	0	N.D	50e	55e	N.D
Sx22	328.1	0	N.D	N.D	30e	N.D
Sx23	387.0	0	N.D	N.D	50e	N.D

N.D. = not done

e = estimate by eye not by densitometry

Table 5.3 continued.

Chimaera no.	Weight of conceptus (mg)	% Pigment (Est. 1)	% Pigment (Est. 2)	%GPI-1B		
				Tail	YS	Placenta
(b) Conceptuses with variegated or pigmented eyes and both GPI-1B and GPI-1C: *b/b* ↔ *c/c* or *b/m* ↔ *c/c* chimaeras						
Sx9	382.7	1	N.D	0.0	7.0	N.D
Sx24	348.5	0	0.79	0.0	15.0e	N.D
Sx10	356.2	1	N.D	0.0	15.2	N.D
Sx58	357.8	10	N.D	0.0	25.3	20.0e
Sx53	320.6	2	1.77	8.5	9.3	38.6
Sx15	367.2	1	N.D	10.0	16.9	N.D
Sx57	336.2	20	N.D	10.1	27.0	0
Sx51	378.7	5	N.D	11.1	48.7	25.3
Sx49	341.6	10	N.D	12.9	70.5	40.1
Sx19	317.7	50	N.D	13.9	79.5	N.D
Sx48	343.3	80	N.D	14.9	5.2	14.3
Sx59	301.2	30	N.D	17.8	0.0	0
Sx42	277.3	80	N.D	20.0	69.3	0
Sx45	382.9	100	N.D	23.2	64.4	0
Sx52	329.9	80	N.D	27.8	92.6	3.0
Sx55	346.2	100	N.D	32.6	23.0	67.6
Sx44	253.9	90	N.D	32.7	45.8	6.0
Sx41	291.5	45	N.D	36.3	21.3	8.0
Sx4	299.1	5	69.13	37.6	40.6	N.D
Sx35	379.1	100	N.D	42.0	34.1	N.D
Sx33	293.8	90	N.D	45e	14.7	N.D
Sx32	367.3	5	N.D	46.5	14.6	N.D
Sx56	313.1	100	N.D	46.8	32.8	51.9
Sx31	333.3	90	N.D	47.3	24.7	N.D
Sx6	353.3	5	N.D	47.6	66.3	N.D
Sx34	292.9	80	N.D	58.7	33.9	N.D
Sx12	257.1	1	N.D	60.8	3.3	N.D
Sx18	325.1	50	N.D	64.5	70.1	N.D
Sx39	457.1	100	N.D	67.2	71.1	N.D
(c) Conceptuses with apparently variegated eyes (Est 1 only) but only GPI-1B. Possibly non-chimaeric (if eye classification is unreliable) but could be *b/b* ↔ *c/c* or *b/m* ↔ *c/c* chimaeras (with *b/m* or *b/b* >>*c/c*)						
Sx25	256.2	40	N.D	100	100	N.D
Sx5	306.9	5	N.D	100	100	N.D
(d) Conceptuses with fully pigmented eyes and only GPI-1B. Probably non-chimaeric, but could be *b/b* ↔ *c/c* or *b/m* ↔ *c/c* (with *b/b* or *b/m* >>*c/c*)						
Sx16	335.6	100	N.D	100	100	N.D
Sx26	348.6	100	N.D	100	100	N.D

(group (f) in Table 5.4). These can therefore be classed as putative homozygous null chimaeras. Four of these can be considered to be only possible *m/m* ↔*c/c* chimaeras and one is considered to be a probable *m/m* ↔ *c/c* chimaera. These subdivisions were made on the basis of the percentage of eye pigment (as discussed below).

In series (ii) 52 embryos were recovered (Tables 5.5 and 5.6). Offspring of the four expected types (*a/m*↔*c/c*, *b/m*↔*c/c*, *a/b*↔*c/c* and putative *m/m* ↔*c/c*) were found. In this series of chimaeras, 34 of the fetuses recovered were chimaeric by GPI analysis. An A + C GPI result was obtained from 11 of the embryos (Group (a) Table 5.5), 14 of the fetuses recovered gave a B + C result (Group (b) Table 5.5), 9 were of the AB + C type (Group (c) Table 5.5) and 18 of the fetuses recovered gave only a GPI - 1 C result (Groups (d) and (e) Table 5.6). No eye pigmentation was detected in 2 (A + C) fetuses, 3 (B + C) fetuses or 1 (AB + C) fetus. Of the 18 fetuses that were GPI-1C only, 10 had albino eyes (group (d) in Table 5.6). These fetuses were probably non-chimaeric (*c/c*) but some could be chimaeric (*a/b* ↔*c/c*, *a/m* ↔*c/c*, *b/m* ↔*c/c* and *m/m* ↔*c/c*) with the (*a/m* x *b/m*) component of the chimaera contributing very little to the tissues examined and therefore below the detection limit of the GPI assay system. The remaining 8 fetuses (group (e) in Table 5.6) are putative homozygous GPI null chimaeras as they are GPI-1C only and have pigment present in the eye. These have been subdivided as 5 possible and 3 probable *m/m* ↔*c/c* chimaeras as discussed below.

Series (iii) is the control series where no homozygous GPI null chimaeras would be expected. Twenty four chimaeras were recovered. Of these, 23 embryos were of the B + C type as would be expected from this series (see Table 5.2). Three of the GPI-1 B + C fetuses had no eye pigmentation (Group (a) in Table 5.7), this may be due to the GPI-1B component of the chimaera not colonising the eye of the fetus. The 20 other chimaeras in this group had pigment varying from 5.3 - 97.3% (group (b) in Table 5.7). One conceptus, (Tx 14, Table 5.7) produced only GPI-1C and had no eye

Table 5.4 Results from series (i) (*b/m* x *b/m*) ↔ *c/c* conceptuses continued. Conceptuses with only GPI-1C (includes putative *m/m* ↔ *c/c* chimaeras):

Chimaera no.	Weight of conceptus (mg)	% Pigment (Est. 1)	% Pigment (Est. 2)	%GPI-1B		
				Tail	YS	Placenta
(e) Conceptuses with albino eyes and only GPI-1C: Probably non-chimaeric but could be *b/b*↔ *c/c* or *b/m* ↔ *c/c* chimaeras (with *c/c*>> other genotypes)						
Sx43	342.3	0	0	0	0	0
Sx 1	317.0	0	0	0	0	N.D.
Sx 2	274.9	0	0	0	0	N.D.
Sx29	244.8	0	0	0	0	N.D.
Sx30	310.9	0	0	0	0	N.D.
Sx36	275.3	0	0	0	0	N.D.
Sx38	331.5	0	0	0	0	N.D.
Sx13	316.9	1	0	0	0	N.D.
Sx47	335.6	10	0	0	0	0
(f) Conceptuses with variegated eyes but only GPI-1C: *b/b* ↔ *c/c* , *b/m* ↔ *c/c* (with *c/c*>> other genotypes) or *m/m* ↔ *c/c* Possible m/m ↔ c/c chimaeras						
Sx40	326.0	0	1.04	0	0	N.D.
Sx50	322.9	0	1.15	0	0	0
Sx46	286.6	10	2.04	0	0	0
Sx28	320.8	0	2.19	0	0	N.D.
Probable *m/m* ↔*c/c* chimaeras*						
Sx11	353.0	1	12.96	0	0	N.D.

* Of the 5 chimaeras in group (f) chimaera Sx 11 is the most likely to be a genuine *m/m* ↔ *c/c* chimaera because the significant contribution of the (*b/m* x *b/m*) genotypic component to the fetal eye (pigmented cells) was not reflected by the presence of GPI-1B in the tail or yolk sac.

pigment present. As with the fetuses of this type in the other series, this fetus was probably non-chimaeric but might have been chimaeric with the proportion of the cells from the (GN x NUL) component being too small for the GPI assay system to detect.

As stated earlier, putative homozygous GPI null chimaeras would be those conceptuses that were GPI-1C only in all of the tissues analysed by GPI electrophoresis but had pigmented cells in the eye. In series (i), 5 chimaeras were identified as this type (group (f) Table 5.4), and in series (ii), 8 chimaeras were GPI-C only and had eye pigment present (group (e) Table 5.6). As expected, none of the chimaeras recovered in control series (iii) were of this type (Table 5.7).The proportion of eye pigment observed in these putative homozygous GPI null chimaeras tended to be low, with 9 having less than 5% pigment detected, 3 with between 10-13% and one with approximately 53% present (Table 5.4 & Table 5.6).

It is difficult to distinguish between the chimaeras that are in fact homozygous GPI null chimaeras and those that are one of the other chimaera types (a/*b*↔*c*/c, *b*/*m* ↔*c*/*c*, *a*/*m* ↔ *c*/*c*), in which the *c*/*c* cell population was overwhelmingly predominant. The assay system could always detect 3% of GPI-1B in artificial mixtures of fetal homogenates (Table 5.1). In the control series of chimaeras, 1/24 fetuses produced only GPI-1C (in the tail) when pigment was present in the eye (Table 5.7). Also none of 178 (albino GPI-1A ↔ pigmented GPI-1B) chimaeras produced in our laboratory for other studies (West, Flockhart, & Kissenpfennig, 1995, West, Flockhart & Keighren 1995 and West & Flockhart, 1994), had only GPI-1A present in the fetal trunk when the eye contained pigmented cells. As already noted, another study has shown a close correlation between the estimated % pigmented cells in a single mid section of retinal epithelium and the corresponding % GPI-1B in the fetus of GPI-1A ↔ GPI-1B fetal chimaeras (Hodson, Keighren & West , unpublished), where the GPI-1B is the pigmented component. These

Table 5.5 Results from series (ii) (*a/m* x *b/m*) ↔ *c/c* conceptuses. Conceptuses with GPI-1A and/or GPI-1B enzyme. Chimaeras are ranked in order of % pigment (Est. 2) and % GPI-1A, GPI-1B or GPI-1AB in the tail.

Chimaera no.	Weight of conceptus (mg)	% Pigment (Est. 1)	% Pigment (Est. 2)	%GPI-1A		
				Tail	YS	Placenta
(a) *a/m* ↔ *c/c* chimaeras						
Vx24	335	0	0	0	17.3	2.5
Vx42	357	1	0	0	9.7	0
Vx27	378	2	2.1	8.9	13.4	17.7
Vx29	367	0	11.36	26.3	70.0	36.3
Vx11	359	25	17.15	5.2	1.1	0.9
Vx2	281	20	21.61	13.7	79.9	0
Vx21	356	70	24.20	23.0	0	44.0
Vx14	351	30	27.65	12.3	6.7	0
Vx1	430	70	29.99	23.2	38.9	29.0
Vx34	413	100	37.07	31.5	20.5	23.5
Vx35	403	100	52.74	33.5	6.4	33.4
(b) *b/m* ↔ *c/c* chimaeras						
				% GPI-1B		
				Tail	YS	Placenta
Vx19	337	0	0	15.0	20e	7.0
Vx40	420	2	0	17.4	72.5	29.6
Vx52	350	70	0	42.0	25.4	38.9
Vx25	329	20	6.50	16.9	35.9	3.2
Vx49	342	4	6.53	11.5	11.8	0
Vx46	233	5	7.57	5.10	16.3	0
Vx44	340	10	8.00	17.9	7.2	4.5
Vx4	283	1	10.19	10.0	89.9	48.2
Vx9	325	35	21.93	18.8	70.3	0
Vx28	288	20	24.25	25.9	41.4	0
Vx50	376	50	28.14	28.4	20.3	41.3
Vx39	419	80	54.49	19.1	34.9	0
Vx41	374	80	60.51	41.0	37.9	33.8
Vx8	277	100	93.42	76.8	58.5	34.5

Table 5.5 continued.

Chimaera no.	Weight of conceptus (mg)	% Pigment (Est. 1)	% Pigment (Est. 2)	%GPI-1(A + AB + B)		
				Tail	YS	Placenta
(c) *a/b* ↔ *c/c* chimeras						
Vx15	267	0	0	55.0	0	0
Vx13	356	50	20.83	21.2	73.0	59.0
Vx30	358	80	26.19	54.8	68.9	48.6
Vx38	414	30	28.37	10.0	74.0	10.2
Vx36	394	100	36.40	14.0	87.5	48.3
Vx18	292	80	51.38	48.2	50.8	69.9
Vx48	324	90	73.57	83.0	N.D	15.9
Vx20	335	100	84.54	85.0	86.0	71.3
Vx16	376	90	84.90	70.4	69.0	43.8

observations suggest that if eye pigment is detectable then the corresponding GPI allozyme should be detectable in another part of the fetus. In this case we would expect to detect 3% GPI-1A, GPI-1AB or GPI-1B so we would expect to detect the (GN x NUL) GPI-1 phenotype if the (GN x NUL) component contributed at least 3% to the eye (≥ 3% pigmented). However, although the correlation between eye pigment and % GPI is very strong, it is not exact. Also, only part of the fetus (tail) was sampled for GPI analysis. As the proportion of eye pigmentation increases, the likelyhood increases that these chimaeras are homozygous GPI null (*m/m↔c/c*) chimaeras. If we adopt a more conservative threshold and consider chimaeras with only GPI-1 C but < 4% eye pigment as merely possible *m/m ↔c/c* chimaeras and those with ≥4% eye pigment as probable *m/m ↔c/c* chimaeras, this produces 9 possible and 4 probable *m/m ↔c/c* chimaeras (Tables 5.4 group (f) and 5.6 group (e). If 3% pigment is adopted as the threshold the number of probable *m/m ↔c/c* chimaeras increases to six.

5.4 DISCUSSION

The aim of this part of the chimaera study was to try to identify which, if any of the embryos recovered were homozygous GPI null chimaeras (*m/m ↔ c/c*) rather than non chimaeric GPI-1C embryos (*c/c*). Looking for the absence of an enzyme rather than its presence poses several problems. Absence of the enzyme may reflect insufficient sensitivity to detect small amounts that are present, or other technical problems such as incorrect sample loading and consequent absence of sample. If a completely negative result was obtained after GPI electrophoresis the sample was always rerun. If the embryo was chimaeric there should always be GPI-1C present from the normal component of the chimaera as long as the assay system was sensitive enough to detect it. An assessment of the sensitivity of the GPI electrophoresis system was undertaken to identify the lowest proportion of GPI-1B present that could be detected. This study suggested that 3% GPI-1B could readily be

Table 5.6 Results from series (ii) (*a/m* x *b/m*) ↔ *c/c* conceptuses continued. Conceptuses with only GPI-1C (includes putative *m/m* ↔ *c/c* chimaeras)

Chimaera no.	Weight of conceptus (mg)	% Pigment (Est. 1)	% Pigment (Est. 2)	%GPI-1(A + AB + B) Tail	YS	Placenta
(d) Conceptuses with albino eyes and only GPI-1C: Probably non-chimaeric but could be *a/m* ↔ *c/c* , *b/m* ↔ *c/c* or *m/m* ↔ *c/c* (with *c/c* >> other genotypes)						
Vx17	330	0	0	0	0	0
Vx3	358	0	0	0	0	0
Vx7	322	0	0	0	0	0
Vx10	365	0	0	0	0	0
Vx22	244	0	0	0	0	0
Vx23	188	0	0	0	0	0
Vx32	286	0	0	0	0	0
Vx43	315	0	0	0	0	0
Vx12	356	0	0	0	0	0
Vx6	260	0	0	0	0	0
(e) Conceptuses with variegated eyes but only GPI-1C: *a/m* ↔ *c/c* , *b/m* ↔ *c/c* (with c/c >> other genotypes) or *m/m* ↔ *c/c* Possible *m/m* ↔ *c/c* chimaeras						
Vx31	229	0	1.58	0	0	0
Vx45	341	0	2.00	0	0	0
Vx5	301	2	2.30	0	0	0
Vx47	273	1	3.32	0	0	0
Vx33	349	0	3.83	0	0	0
Probable *m/m* ↔ *c/c* chimaeras*						
Vx37	362	5	10.30	0	0	0
Vx26	273	0	12.10	0	0	0
Vx51	312	0	52.80	0	0	0

* Of the 8 fetuses in group (e), the last 3 are most likely to be genuine *m/m* ↔ *c/c* chimaeras because the significant contribution of the (*a/m* x *b/m*) genotypic component to the fetal eye (pigmented cells) was not reflected by the presence of GPI-1A, GPI-1AB or GPI-1B in the tail, yolk sac or placenta.

detected if present within an embryo. There is no simple way to identify a homozygous GPI null embryo at the 8 - cell stage so that they could be used to produce fetal aggregation chimaeras because maternally derived enzyme persists beyond this stage (West & Green, 1983). A two step analysis was performed. Firstly non-chimaeric fetuses were identified and discounted by lack of eye pigment. Secondly chimaeric fetuses were distinguished by GPI electrophoresis. Following both these analyses, 46 chimaeras were identified in series (i), 42 in series (ii) and 23 in series (iii). In series (iii) as would be expected none of the chimaeras were homozygous GPI null chimaeras. However in series (i) and series (ii), 5 and 8 respectively were identified as putative homozygous GPI null chimaeras.

Having identified putative *m/m* ↔ *c/c* chimaeras, it is necessary to try to determine whether they are likely to be genuine homozygous GPI null chimaeras or simply other chimaeras (eg *b/m* ↔ *c/c*) in which the *c/c* component predominates. The likelihood that a fetus, that has eye pigmentation but only GPI-1C, is a homozygous GPI null chimaera increases with the percentage pigmentation observed. The GPI electrophoresis system should normally detect 3% or more GPI-1B (or GPI-1A or GPI-1AB). Since the % eye pigmentation and % GPI correlated well in another study, fetuses with greater than 3% eye pigmentation but no GPI are likely to be *m/m* ↔*c/c* chimaeras. None fell in this class in control series (iii).

There may be other putative homozygous GPI null chimaeras in this study that are GPI-1C only and albino in the eye, that have not been identified. In the 3 series of chimaeras produced, a number of fetuses were identified by GPI type as chimaeric, but had no detectable pigment in the eye (Table 5.3, 5.5 and 5.7). Some of these will be investigated further in Chapter 6 (Sx14, Sx37 and Sx54 from series (i), Vx19, Vx24 and Vx40 from series (ii) and Tx9 and Tx4 from series (iii)). If the homozygous GPI null cell population was similarly excluded from the eyes of any *m/m* ↔ *c/c* chimaeras they would not have been distinguished fom the non-

Table 5.7 Results from control series (iii) (*b/m* x *b/b*) ↔ *c/c* ranked in order of % pigment (Est 2) and % GPI-1B in the tail.

Chimaera no.	Weight of conceptus (mg)	% Pigment (Est. 1)	% Pigment (Est. 2)	%GPI-1B		
				Tail	YS	Placenta
(a) Conceptuses with albino eyes but both GPI-1B and GPI-1C: *b/b* ↔ *c/c*, or *b/m* ↔ *c/c* chimaeras						
Tx4	336.2	0	0	0	0	4.3
Tx9	343.5	0	0	0	2.9	54.2
Tx17	415.0	0	0	0	30.8	0
(b) Conceptuses with variegated or pigmented eyes and both GPI-1B and GPI-1C: *b/b* ↔ *c/c*, or *b/m* ↔ *c/c* chimaeras						
Tx5	330.4	2	5.30	14.5	0	18.7
Tx2	327.9	35	8.52	7.9	10.4	2.2
Tx16	405.1	10	9.06	18.8	71.2	7.8
Tx23	309.8	5	10.7	10.0	69.1	40.2
Tx6	350.7	40	11.7	0	8.1	0
Tx3	351.4	10	15.6	10.9	12.4	3.7
Tx19	410.0	25	16.6	7.3	70.7	14.9
Tx7	322.6	40	20.2	5.1	2.1	0
Tx20	443.6	50	22.1	14.2	5.0	5.8
Tx11	319.9	50	26.3	25.4	14.6	0
Tx12	340.0	50	28.4	17.2	0	20.1
Tx1	318.0	100	61.3	25.4	21.9	23.7
Tx15	413.8	100	67.2	41.4	59.3	31.7
Tx13	329.6	100	68.2	50.2	93.4	24.3
Tx22	330.8	100	71.2	50.4	30e	59.4
Tx8	319.0	100	75.9	46.7	18.3	47.8
Tx21	337.9	60	76.1	38.5	37.0	25.5
Tx10	273.9	90	81.4	58.1	52.6	19.2
Tx24	315.8	90	83.4	49.8	26.7	38.5
Tx18	411.3	100	97.3	57.6	60.4	16.9
(c) Conceptuses with albino eyes and only GPI-1C. Probably non chimaeric but could be *b/b* ↔ *c/c*, or *b/m* ↔ *c/c* chimaeras (with *c/c*>>other genotypes)						
Tx14	257.9	0	0	0	0	0

chimaeric albino, GPI-1C only fetuses because the null allele cannot be detected by GPI electrophoresis. If pigment was present at a very low level it might not have been detected. As already mentioned, the first estimate (Est 1) was somewhat subjective. The second estimate was based on a histological section cut from the middle of the eye. Although several flanking sections were also checked if the mid-section was entirely albino, the entire eye was not always examined. The second eye was not examined for either Est 1 or Est 2. Any errors here would serve to underestimate the number of *m/m* ↔ *c/c* chimaeras, so does not invalidate the conclusion that *m/m* cells can be rescued in aggregation chimaeras. Thus, the question posed in the title to this Chapter can be answered: homozygous null cells can be rescued in aggregation chimaeras.

The analysis of putative chimaeric conceptuses with GPI and pigment markers described in this Chapter identified 13 putative *m/m* ↔ *c/c* chimaeras (possible and probable *m/m* ↔ *c/c* chimaeras). These together with 19 probable non-chimaeric *c/c* conceptuses, were analysed further by *in situ* hybridisation to the transgenic marker. This analysis was designed to refine the classification of *m/m* ↔ *c/c* chimaeras and is described in the next Chapter.

CHAPTER 6

FURTHER IDENTIFICATION OF FETAL HOMOZYGOUS NULL CHIMAERAS.

6.1 INTRODUCTION

The previous Chapter identified thirty two, 12.5 day conceptuses from series (i) and (ii) that were likely to be either *m/m* ↔ *c/c* chimaeras or non-chimaeric *c/c*. This Chapter describes analysis of these conceptuses by *in situ* hybridisation to a transgenic marker that was used to refine the identification of probable homozygous null chimaeras.

The NUL males were homozygous for a reiterated β globin transgenic marker, therefore all chimaeric offspring will be hemizygous for the transgenic marker. A DNA:DNA in situ hybridisation study was undertaken to look for the presence of transgenic cells in certain areas of the chimaeric conceptus (brain, tail, amnion, yolk sac mesoderm, yolk sac endoderm and placenta, see Fig 6.1). All 32 conceptuses that produced 100% GPI-1C and another 20 (6 from series (i), 6 from series (ii) and 8 from control series (iii)) were analysed. The control chimaeras (non-*m/m* ↔ *c/c*) in this study were selected for the presence of low proportion of eye pigmentation (which corresponds to low proportion of (GN x NUL) transgenic cells in the fetus) to compare with the probable homozygous null chimaeras (which were observed to have low proportions of eye pigmentation).

6.2 MATERIALS AND METHODS.

For explanation of Chimaera Production and processing for Histology see Chapter 5.2 and Appendices VI, VII and VII.

TRANSGENIC MARKER

The NUL males used to produce the pigmented component of the chimaeras are homozygous for a transgene (TgN(Hbb-b1)83Clo, which will be referred to as Tg in this thesis) comprising approximately 1000 tandemly repeated copies of a mouse β globin transgene inserted on chromosome 3 (Lo, 1986).

LABELLING DNA PROBES WITH DIGOXYGENIN.

Labelling of DNA probes with digoxygenin was carried out by Mrs. Margaret Keighren, Department of Obstetrics and Gynaecology, University of Edinburgh.

The β globin probe is derived from the plasmid (pMBJ) which was inserted into the strain 83 transgenic mice (Lo, 1986). The plasmid pMßð2 was linearised by incubation at 37°C with EcoRI (1 units/μg) for 90min. A sample was run on a 0.8% agarose gel to confirm that restriction had taken place. Phenol/chloroform extraction was performed and the DNA was ethanol precipitated overnight at -20°C. The precipitate was dissolved in TE buffer and stored as a stock of 100ng/μl. The probe was denatured by boiling 1μg of DNA in distilled water in a total volume of 10μl, then removing to ice. The labelling reaction was carried out using reagents from the Boehringer Mannheim DIG DNA Labelling and Detection Kit. To the total volume of 10μl was added 2μl of 10x hexanucleotide mixture and 2μl of 10 x dNTP labelling mixture.The reaction mixture was incubated at 37°C for 6hr with 2U/μl of Klenow enzyme (1μl), then stopped by the addition of 2μl of 0.2M EDTA pH 8. The labelled DNA was precipitated overnight at -20°C with 60 ml cold ethanol and 2μl of 3M sodium acetate (see Keighren & West, 1993). The labelled DNA was then spun

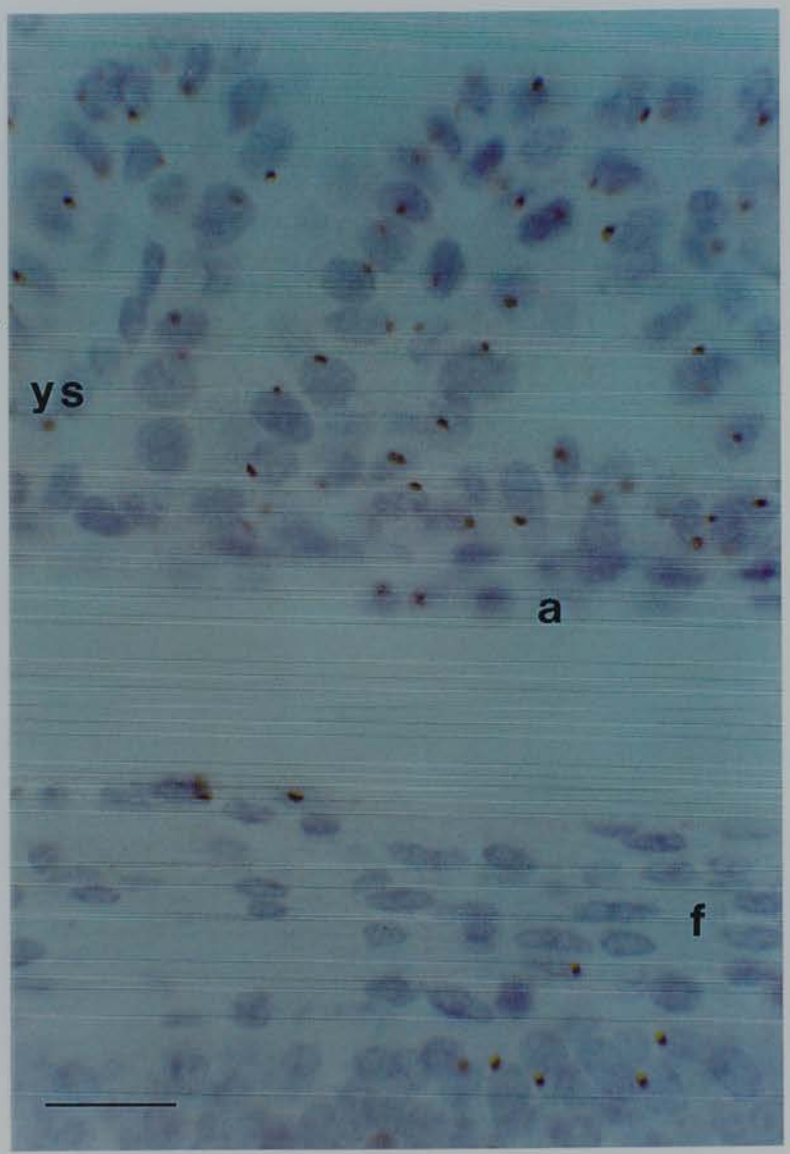


Fig 6.1 Tissues sections following *in situ* hybridisation to the β-globin transgenic marker. Dark spots in the nucleus of cells indicates the prescence of the transgene.Scale bar = 20μm.
ys = yolk sac, f=fetus, a = amnion

down and dried under vacuum, then redissolved in 50µl of TE buffer to give a final stock concentration of 20 ng/µl of labelled DNA. In later stages of the project nick translation was used to label the probe (see Appendix III (c)).

IN SITU HYBRIDISATION ANALYSIS

Tissue sections were mounted on TESPA-coated slides, dried and dewaxed in Histoclear (National Diagnostics) (2 x 10mins), and rehydrated (2 x 5min in absolute ethanol, 15-30 mins in 3% H_2O_2 in methanol to remove endogenous peroxidase activity followed by 2 x 5mins in 70% ethanol and 2 x 5mins in PBS). The tissue DNA was then denatured by placing the slides in 1M NaOH for 3min at 70°C and washed in cold PBS at 4°C. The sections were then prehybridised. 40µl of the prehybridisation mixture (see Appendix III(c)) was placed on a glass coverslip and picked up onto a slide. The slides were then placed inside a preheated humidified chamber and incubated at at 60°Cfor 15 mins. Hybridisation was carried out at 60°C overnight. 40µl per slide of the hybridisation mixture (see Appendix II(c)) was placed on plastic hydrophobic coverslips (Gel Bond) and picked up by the slide, the coverslips were sealed with nail varnish. The slides were then washed in 2 x SSC containing 0.1% Triton-X (2 x 5min) followed by 0.1 x SSC, 0.1% Triton-X (1 x 5min at room temperature, 1 x 5min at 50°C) and 0.1x SSC, 0.1 Triton-X with 5% BSA (1 x 5min) (see Appendix III(c)) and Buffer 1 (1 x 5mins) (see Appendix III(c)). The slides were then incubated with 1:100 dilution of anti-digoxigenin HRP-antibody (see Appendix III(c)) in Buffer 1 for 30min in a dark humidified chamber. At the end of this treatment the slides were washed in two changes of Buffer 1 for 10 min each, followed by a 5 min wash in 50 mM Tris[pH7.3] (DAB buffer, see Appendix III (c)). The slides were then flooded with development reagent for 40 mins in a humidified box. The slides were then washed in water, stained for 15-20secs in haematoxylin, 15secs in acid/alcohol and 10-20 secs in Scotts tap water (see Appendix III(c)). Preliminary inspection of the sections at this stage ensured

that there was enough contrast between the *in situ* marker and the nuclear counterstain so that the "spots" (Fig 6.1) could be distinguished. The slides were then stained with eosin for 5secs then dehydrated through a series of alcohols (70%, 85%, 90% and 100% ethanol for 30s each), immersed in Histoclear for 3mins and mounted in Histomount (National Diagnostics) (see Keighren & West, 1993).

6.3 RESULTS

The NUL males are homozygous for the reiterated β globin transgene, therefore all the chimaeric offspring should have a cell population that is hemizygous for the transgene. Another study (West, Flockhart & Keighren, unpublished) has shown that hemizygous transgenic cells are not at a selective disadvantage in Tg/- ↔ -/- chimaeras. The proportion of cells with the transgene corresponds to the proportion of (GN x NUL) cells in the chimaera. Therefore, in the homozygous GPI null chimaeras (*m/m*, Tg/- ↔ *c/c*, -/- chimaeras), the homozygous GPI null cells are transgenic and will be detected by *in situ* hybridisation.

Approximately 300 cells were scored for the presence of the transgene in each region examined (placenta, yolk sac endoderm, yolk sac mesoderm, amnion, tail region and brain) of all 52 conceptuses analysed. The crude % of Tg positive cells was corrected to allow for the observation that only 72% of cells were positive in hemizygous Tg/- fetal heads.

In Chapter 5, a threshold value of ≥4% eye pigmentation in the absence of GPI-1A or GPI-1B was used to identify probable homozygous null chimaeras. The (GN x NUL) cells give rise to the pigment and also carry the β globin transgenic marker. Therefore the 4% threshold was also applied to the proportion of transgenic cells detected in this study and was used to try to identify probable homozygous null chimaeras. However it was only used for the tissues previously sampled for GPI electrophoresis, that is the tail, yolk sac and placenta. In this study the yolk sac

Table 6.1. The % transgene detected in the tissues analysed of chimaeric fetuses from series (ii) that were classified in Chapter 5 as either *a/m* ↔ *c/c* or *b/m* ↔ *c/c* (N=6). Ranked in order of % pigment (Est 2) and % transgene detected in the brain.

Chimaera no.	% Pigment Est 2	Corrected % Transgene detected					
		Brain	Tail	Amnion	YS mes	YS end	Placenta
Vx24	0	0.00	0.00	0.00	0.00	0.41	0.00
Vx40	0	3.15	0.44	11.82	2.84	11.57	65.74
Vx19	0	26.33	0.00	0.39	0.00	17.96	0.00
Vx27	2.11	1.40	26.47	12.04	9.06	42.70	21.02
Vx49	6.53	5.45	13.27	6.12	3.56	0.39	12.28
Vx46	7.57	5.61	8.22	3.04	0.46	18.52	7.26

mesoderm and yolk sac endoderm were examined separately, whereas the GPI analysis was done using whole yolk sac. Therefore a mean value of 4% transgenic cells detected in the yolk sac mesoderm and yolk sac endoderm was used as the threshold. A sample of placenta was not taken for GPI analysis from the first 40 chimaeras produced in series (i) (conceptuses Sx1 - Sx40), so the 4% threshold was not applied to the placenta of these chimaeras either (14 included in the *in situ* hybridisation analysis).

The results from the *in situ* analysis are shown in Tables 6.1 - 6.4. Table 6.1 shows the results from the *a/m* ↔*c/c* and *b/m* ↔*c/c* chimaeric conceptuses from series (ii) that were analysed (N=6). These chimaeras carry one copy of the *Gpi-1s* null allele. The results from the *in situ* analysis of the *b/b* ↔*c/c* or *b/m*↔*c/c* chimaeras (N=14) are shown in Table 6.2. It is unknown which of these chimaeras have either one copy of the null allele or two normal *Gpi-1s* alleles.

Table 6.3 and Table 6.4 show the results from the transgenic analysis of the conceptuses that were reclassified as possible and probable homozygous null chimaeras respectively. Four chimaeras were identified as non-chimaeric because they produced GPI-1C only, lacked eye pigment and had no detectable transgene in any of the areas analysed (Table 6.3 group (a)) and 2 chimaeras had low levels of eye pigment detected but no transgene present in the tissues examined (Table 6.3 group (b)). Using 4% transgenic cells as a threshold to reclassify the possible and probable null chimaeras, has identified 14 chimaeras as possible homozygous null chimaeras (Table 6.3(b), (c) and (d)) with the proportion of transgene detected being below the 4% threshold value and 14 chimaeras as probable homozygous null chimaeras with the proportion of transgene detected being above the 4% threshold limit (Table 6.4).

Table 6.2. The % transgene detected in tissues analysed from chimaeras of series (i) and (iii) that were classified in Chapter 5 as either *b/m* ↔ *c/c* or *b/b* ↔ *c/c* (N=14). Conceptuses are ranked in order of % pigment (Est 2) and % trangene detected in the brain.

Chimaera no.	% Pigment Est 2	Corrected % Transgene detected					
		Brain	Tail	Amnion	YS mes	YS end	Placenta
(a) Conceptuses with albino eyes. Either *b/m* ↔ *c/c* or *b/b*↔*c/c* chimaeras							
Sx37	0	0.00	0.00	0.43	0.42	0.00	0.39
Sx14	0	0.00	0.79	0.00	0.00	3.08	33.08
Tx9	0	0.00	0.47	1.01	0.00	20.15	55.01
Tx4	0	0.88	0.00	0.00	0.00	0.00	14.54
Tx14	0	0.45	0.00	0.52	0.00	0.41	0.47
Sx54	0	6.61	12.66	1.06	0.43	12.35	3.58
(b)Conceptuses with variegated eyes. Either *b/m* ↔*c/c* or *b/b*↔*c/c* chimaeras.							
Sx24	0.79	0.45	0.47	1.61	0.45	17.36	0.00
Sx53	1.77	0.00	3.49	0.46	0.00	1.85	59.14
Tx5	5.30	3.27	7.02	3.77	0.41	34.72	85.50
Tx16	9.06	4.83	0.00	3.48	5.98	44.61	27.87
Tx23	10.70	16.67	7.83	1.31	1.86	56.44	62.17
Tx3	15.60	4.03	12.43	10.49	7.94	62.29	10.12
Tx7	20.20	0.00	0.00	0.00	0.00	42.63	0.39
Sx4	69.13	33.37	62.39	22.11	6.92	31.94	65.08

6.4 DISCUSSION

The *in situ* hybridisation analysis enabled the reclassification of some possible homozygous nulls (and some probable non-chimaeras) to probable homozygous nulls using a 4% threshold value for the tissues that were also analysed by GPI electrophoresis. The group of 4 probable homozygous null chimaeras identified in Chapter 5 has now been increased to 14. Six of these were from series (i) and 8 were from series (ii). It also identified 4 non-chimaeric conceptuses (one from series (i) and 3 from series (ii))

In series (i) 5/59 (8.5%) conceptuses appeared to be non-chimaeric (one conceptus that produced GPI-1C only and 4 conceptuses that produced GPI-1B only). Of the remainder, 41/54 were either *b/b* ↔*c/c* or *b/m* ↔*c/c*, 6/54 (11.1%) were probable *m/m*↔*c/c* chimaeras (the remaining 7 were classed as possible *m/m* ↔*c/c* chimaeras). The expected frequency of *m/m*↔*c/c* chimaeras was 25%. The ratio of 6:48 is significantly lower than the predicted 1:3 ratio (χ^2 = 5.56; P<0.025). However, if all the possible and probable *m/m*↔*c/c* chimaeras are genuine (13/54), there is no significant difference from the number of *m/m*↔*c/c* chimaeras expected (χ^2 = 0.025; P>0.05). Comparison of the number of moles recovered at 12.5 days in series (i) and control series (iii) showed no significant difference (Table 6.5; 21:59 versus 4:24; χ^2=1.06; P=0.30) suggesting that a significantly higher proportion of homozygous GPI null chimaeras is not lost before 12.5 days. The proportion of non-chimaeras (100% GPI-1C: chimaeric: 0% GPI-1C; 1:54:4) in this series was also not significantly higher than in the control series (iii), (0:24:0) (χ^2=2.16; P=0.339) so there is no evidence that any *m/m*↔*c/c* chimaeras were misclassified as non-chimaeric.

In series (ii) 3/52 (5.8%) conceptuses appeared to be non-chimaeric (the 3 conceptuses produced 100% GPI-1C only, and none were 0% GPI-1C) which was

Table 6.3 Reclassification of conceptuses as *c/c* non-chimaeras or possible *m/m* ↔ *c/c* chimaeras (only GPI-1C and >4% pigment or Tg/- cells)

Chimaera no.	% Pigment Est 2	Corrected % Transgene detected					
		Brain	Tail	Amnion	YS mes	YS end	Placenta
(a) Conceptuses with albino eyes and only GPI-1C with no transgenic cells detected. Therefore non-chimaeric.							
Sx43[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vx17[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vx22[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vx43[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(b) Conceptus wth variegated eyes and GPI-1C only, but with no detectable transgene in tissues typed for GPI. Possible *m/m* ↔ *c/c* but could also be *a/m* ↔*c/c*, *b/m*↔*c/c*, *b/b* ↔c/c or (where *c/c*>>other genotypes)							
Vx31[b]	1.58	0.00	0.00	0.00	0.00	0.00	0.00
Sx46[b]	2.04	0.00	0.00	0.00	0.00	0.00	0.00
(c) Conceptuses with albino eyes and GPI-1C only, but with transgene detected (% Tg/- cells <4% in tissues which produced only GPI-1C) Possible *m/m* ↔*c/c* (where c/c>>*m/m*)							
Sx29[a]	0	ND	0.00	0.00	0.00	3.26	31.50*
Sx1[a]	0	0.00	0.00	0.00	0.00	0.43	0.00
Sx2[a]	0	0.00	0.00	0.00	0.00	3.60	0.00
Vx6[a]	0	0.00	0.00	0.91	2.42	0.43	0.00
Vx3[a]	0	0.00	0.00	0.00	0.00	3.55	0.00
Vx12[a]	0	0.00	0.00	0.52	0.00	0.00	0.00
Vx7[a]	0	0.46	0.00	0.39	0.00	0.00	0.00
Sx47[a]	0	0.46	2.06	4.93	0.43	0.00	0.47
Vx32[a]	0	0.86	0.84	1.82	0.79	7.07	2.21
Sx30[a]	0	1.12	0.00	0.00	0.00	0.45	0.39
(d) Conceptuses with variegated eyes and GPI-1C only with transgene detected. Possible *m/m* ↔*c/c* chimaeras							
Sx40[b]	1.04	0.53	1.65	2.03	0.00	0.00	20.90*
Vx47[b]	3.32	4.38	2.13	ND	0.00	0.00	0.00

Although >4% Tg/- cells were present in the placenta, this chimaera is not classed as a probable *m/m* ↔*c/c* chimaera because GPI was not analysed in the placenta.

[a] Originally classed as probably non-chimaeric (c/c) conceptuses in Chapter 5.

[b]Originally classed as possible *m/m* ↔*c/c* chimaeras in Chapter 5.

*Although >4% Tg/- cells were present in the placenta, this chimaera is not classified as a probable*m/m* ↔ *c/c* chimaera because GPI was not analysed in the placenta.

not significantly different from either the control series (iii) (3:49:0 versus 0:24:0; $\chi^2 = 1.44$; P=0.23) or series (i) (3:49:0 versus 1:54:4; $\chi^2 = 4.82$: P= 0.09). The observed ratio of 9 *a/b*↔*c/c* : 11 *a/m*↔*c/c*: 14 *b/m*↔*c/c*: 15 (possible and probable) *m/m*↔*c/c* chimaeras is close to the expected 1:1:1:1 ratio (χ^2=1.86; P>0.05). The frequency of *a/m*↔*c/c* plus *b/m*↔*c/c* heterozygous null chimaeras compared to wild type, *a/b* ↔*c/c* was not significantly different from the 2:1 expected ratio.(25:9 tested against 2:1 expected, $\chi^2 = 0.72$; P>0.05). Overall 25% of the chimaeras would be expected to be *m/m* ↔*c/c* homozygous null chimaeras. If only the 8 probable *m/m* ↔*c/c* chimaeras are considered the ratio 8:41 is not significantly different from the expected 1:3 ratio ($\chi^2 = 1.97$; P>0.05). When the number of moles recovered at 12.5 days is compared in series (ii) and (iii), there is no significant difference (14:52 versus 4:24; χ^2=0.244; P=0.621) which again suggests that the homozygous null embryos are not lost before 12.5 days. Neither series shows an excess of *m/m* ↔*c/c* chimaeras if all the possible homozygous null chimaeras are included. This would suggest that identification of these chimaeras is correct and that they have not been misclassified.

The next Chapter describes a more detailed analysis of the heterozygous null (*a/m* ↔*c/c* and *b/m* ↔*c/c*) and homozygous normal (*a/b* ↔*c/c*) chimaeras identified in this Chapter 5. The aim was to determine whether heterozygous null cells were at a selective disadvantage in *a/m* ↔*c/c* and *b/m* ↔*c/c* chimaeras.

Table 6.4 Reclassification of conceptuses as probable *m/m* ↔ *c/c* chimaeras (N=14) (% Tg/- cells ≥4% in tissues which produced only GPI-1C)

Chimaera no.	% Pigment Est 2	Corrected % Transgene detected					
		Brain	Tail	Amnion	YS mes	YS end	Placenta
Probable *m/m* ↔*c/c* chimaeras							
Vx10[a]	0	0.00	0.00	0.00	0.00	0.00	5.45
Sx38[a]	0	0.00	0.00	0.00	0.00	8.19	0.00
Sx36[a]	0	0.00	0.00	0.00	0.00	47.63	0.00
Sx13[a]	0	1.15	0.53	3.67	0.00	10.36	88.18
Vx23[a]	0	1.86	0.53	0.00	19.96	50.50	0.00
Sx50[b]	1.15	1.93	0.00	2.48	0.42	45.34	0.59
Sx28[b]	2.19	4.50	0.00	2.30	0.41	15.82	0.86
Vx45[b]	2.00	0.00	0.00	0.00	0.00	19.74	1.21
Vx5[b]	2.30	1.27	5.30	5.72	0.93	2.61	0.00
Vx33[b]	3.83	1.30	4.96	2.31	0.41	0.38	13.55
Vx37[c]	10.30	10.58	ND	2.25	1.44	6.06	10.62
Vx26[c]	12.10	1.31	7.08	9.54	2.79	4.95	5.86
Sx11[c]	12.96	11.16	12.32	10.33	2.15	20.55	17.25
Vx51[c]	52.82	38.41	38.66	8.64	10.30	9.85	48.06

[a] Originally classed as probably non-chimaeric (c/c) conceptuses in Chapter 5.

[b] Originally classed as possible *m/m* ↔*c/c* chimaeras in Chapter 5.

[c] Originally classed as probable *m/m* ↔*c/c* chimaeras in Chapter 5.

Table 6.5 The fetuses and resorbing moles recovered from embryo transfers in series (i), (ii) and (ii)

	Number (%)		
	series (i)	series (ii)	control series (iii)
Aggregated embryos transferred†	131	86	41
Females with implantations at 12.5days	19	14	6
Total no. of implantations	80 (61%)	66 (77%)	28 (68%)
Normal fetuses	59 (45%)	52 (60%)	24 (58%)
No. chimaeric fetuses	54 (92%)	49 (94%)	24 (100%)
Resorbing moles	21 (26%)*	14 (21%)*	4 (14%)*

†Excluding those transferred to females with no implantation sites
* Expressed as a percentage of total implantations

CHAPTER 7

QUANTITATIVE ANALYSIS OF HETEROZYGOUS NULL AND HOMOZYGOUS NORMAL FETAL CHIMAERAS

7.1 INTRODUCTION

Previous chimaera studies involving genetically abnormal and normal embryos have shown that there is sometimes preferential allocation and survival of the abnormal cells in specific lineages of the resulting conceptus, for example in tetraploid ↔ diploid chimaeras, the tetraploid cells are found in the extraembryonic lineages with little or no contribution to the fetus (Tarkowski & Witkowska, 1977, Nagy *et al*, 1990, James *et al*, 1993).

In chimaeras produced by the aggregation of normal fertilized embryos and parthenogenetic embryos, the parthenogenetic cells were selected against in the extraembryonic tissues but there was an almost normal contribution to the embryo at 12.5d. However this contribution declines as development proceeds to term (Nagy *et al* 1987, Thomson & Solter, 1988b, Fundele *et al*, 1990). In the case of androgenetic ↔ normal fertilized embryos, the androgenetic cells were found mainly in the trophoblast and yolk sac and not in the embryo, the reverse of what is found in parthenogenetic chimaeras (Surani et al, 1988).

The chimaeras analysed in this Chapter are the heterozygous null chimaeras (*a/m* ↔*c/c*, *b/m* ↔ *c/c*) and "homozygous normal" chimaeras (*a/b* ↔*c/c*) from series (ii). Although the *a/b* cells in the *a/b* ↔*c/c* chimaeras are heterozygous at the *Gpi-1s* locus, the *a/b* ↔*c/c* chimaeras will be referred to as homozygous normal chimaeras (i.e. +/+ ↔*c/c*) since both *Gpi-1s*^{a} and *Gpi-1s*^{b} alleles have wild type activity.

The aim was to determine whether the heterozygous null cells (*+/m*) were at a selective disadvantage within chimaeras compared with cells that have no copies of the null allele.

7.2 RESULTS

Before any analysis involving the contribution of GPI-1A (+ GPI-1AB + GPI-1B) to different tissues of the chimaeras was undertaken, the data were corrected to allow for the observation that *Gpi-1s*$^{c/c}$ fetuses produce only 75% of wild type GPI activity (Table 7.1). The % GPI-1A and GPI-1B in the *a/m* ↔*c/c* and the *b/m* ↔*c/c* chimaeras was further revised to allow for the expectation that heterozygous null cells (*a/m* and *b/m*) would have only 50% of wild type activity. Also the contribution of GPI-1C to the placenta was adjusted to allow for 45% of the GPI-C content to be of maternal origin (James *et al* , 1993). Future reference to % GPI-1A (or GPI-1AB or GPI-1B) will be to the corrected values.

HOMOZYGOUS NORMAL CHIMAERAS

Nine homozygous normal (*a/b* ↔*c/c*) chimaeras were produced from series (ii). The distributions of eye pigment (Est 2) and the corrected % GPI-1A (+ GPI-1AB + GPI-1B) in the tail, yolk sac and placenta of these chimaeras was plotted in Fig. 7.1(a,c,e,g). The four distributions are fairly balanced, with the means close to 50%. The contribution of GPI-1A (+GPI-1AB + GPI-1B) to the placenta appears to be bimodal (as reported for other series of chimaeras, James *et al*, 1993; West *et al* 1995) but, overall it is reasonably balanced. The proportion of chimaeras with >50% *a/b* contribution was not significantly different from the expected 1:1 ratio (>50%:<50%) for any of the tissues shown in Fig 7.1 a,c,e,g (χ^2 test).

Relationships in the % GPI-1A (+GPI-1AB + GPI-1B) between pairs of tissues are shown in Fig 7.2 and correlations in Table 7.2. No significantly positive correlations were observed between pairs of tissues in the homozygous normal

Table 7.1 Estimation of the % GPI-1A, GPI-1AB or GPI-1B cells from series (ii).
The observed % GPI in tail, yolk sac and placenta. The observed % GPI is corrected for the observation that the activity of GPI-1C is 75% the activity of wild type GPI and, in the heterozygous null chimaeras (*a/m* and *b/m*), the activity of GPI-1A (or GPI-1B) per cell is likely to be only 50% the activity of *a/a*, *a/b*, or *b/b* cells.

a/b ↔*c/c* chimaeras

Chimaera no	Pigment (Est 2 %)	Observed % GPI-1(A+AB+B)			Corrected % GPI-1(A +AB+B) if C =75% wild type activity			
		Tail	Yolk Sac	Placenta	Tail	Yolk Sac	Placenta	Placenta*
Vx15	0.00	55.00	0.00	0.00	47.83	0.00	0.00	0.00
Vx13	20.83	21.20	73.00	59.00	16.79	66.97	51.91	94.38
Vx30	26.19	54.80	68.90	48.60	47.62	62.43	41.49	75.44
Vx38	28.37	10.00	74.00	10.20	7.69	68.10	7.85	14.27
Vx36	36.40	14.00	87.50	48.30	10.88	84.00	41.20	74.91
Vx18	51.38	48.20	50.80	69.90	41.10	43.64	63.53	115.51†
Vx48	73.57	83.00	ND	15.90	78.55	ND	12.42	22.58
Vx20	84.54	85.00	86.00	71.30	80.95	82.17	65.07	118.31†
Vx16	84.90	70.40	69.00	43.80	64.08	62.54	36.89	67.07

a/m ↔*c/c* chimaeras

Chimaera no	Pigment (Est 2 %)	Observed % GPI-1A			Corrected % GPI-1A if % GPI-1C observed=75% wild type activity			Expected %GPI-1A cells if % GPI-1C observed=50% homozygous wild type activity			
		Tail	Yolk Sac	Placenta	Tail	Yolk Sac	Placenta	Tail	Yolk Sac	Placenta	Placenta*
Vx24	0.00	0.00	17.30	2.50	0.00	13.56	1.89	0.00	23.88	3.70	6.73
Vx42	0.00	0.00	9.70	0.00	0.00	7.46	0.00	0.00	13.88	0.00	0.00
Vx27	0.00	8.90	13.40	17.65	6.83	10.40	13.85	12.78	18.84	24.33	44.24
Vx29	11.36	26.25	70.00	36.30	21.07	63.64	29.94	34.81	77.78	46.09	83.80
Vx11	17.15	5.20	1.10	0.90	3.95	0.83	0.68	7.60	1.64	1.34	2.44
Vx2	21.61	13.70	79.90	0.00	10.64	74.88	0.00	19.23	85.64	0.00	0.00
Vx21	24.20	23.00	0.00	44.00	18.30	0.00	37.08	30.94	0.00	54.10	98.36
Vx14	27.65	12.30	6.70	0.00	9.52	5.11	0.00	17.38	9.72	0.00	0.00
Vx1	29.99	23.20	38.90	29.00	18.47	32.32	23.45	31.18	48.85	37.99	69.07
Vx34	37.07	31.50	20.50	23.45	25.64	16.21	18.68	40.82	27.89	31.48	57.24
Vx35	52.74	33.50	6.40	33.40	27.42	4.88	27.33	43.04	9.30	42.93	78.05

Table 7.1 continued

b/m ↔ *c/c* chimaeras

Chimaera no	Pigment (Est 2 %)	Observed % GPI-1B			Corrected % GPI-1B if % GPI-1C observed =75% wild type activity			Expected %GPI-1B cells if % GPI-1C observed=50% of homozygous wild type activity			
		Tail	Yolk Sac	Placenta	Tail	Yolk Sac	Placenta	Tail	Yolk Sac	Placenta	Placenta*
Vx19	0.00	15.00	20.00	7.00	11.69	15.79	5.34	20.93	27.27	10.14	18.44
Vx40	0.00	17.40	72.50	29.60	13.64	66.41	23.97	24.01	79.82	38.68	70.33
Vx52	0.00	42.00	25.40	38.90	35.20	20.34	32.32	52.07	33.81	48.85	88.82
Vx25	6.50	16.90	35.90	3.20	13.23	29.58	2.42	23.37	45.65	4.72	8.58
Vx49	6.53	11.50	11.80	0.00	8.88	9.12	0.00	16.31	16.71	0.00	0.00
Vx46	7.57	5.10	16.30	0.00	3.87	12.74	0.00	7.46	22.61	0.00	0.00
Vx44	8.00	17.90	7.20	4.50	14.05	5.50	3.41	24.64	10.42	6.60	12.00
Vx4	10.19	10.00	89.90	48.20	7.69	86.97	41.10	14.29	93.03	58.26	105.93†
Vx9	21.93	18.80	70.30	0.00	14.80	63.97	0.00	25.78	78.02	0.00	0.00
Vx28	24.25	25.90	41.40	0.00	20.77	34.63	0.00	34.40	51.45	0.00	0.000
Vx50	28.14	28.40	20.30	41.30	22.93	16.04	34.54	37.30	27.64	51.35	93.36
Vx39	54.49	19.10	34.90	0.00	15.04	28.68	0.00	26.15	44.57	0.00	0.00
Vx41	60.51	41.00	37.90	33.75	34.26	31.40	27.65	51.04	47.79	43.32	78.76
Vx8	93.42	76.80	58.50	34.50	71.29	51.39	28.32	83.24	67.89	44.14	80.25

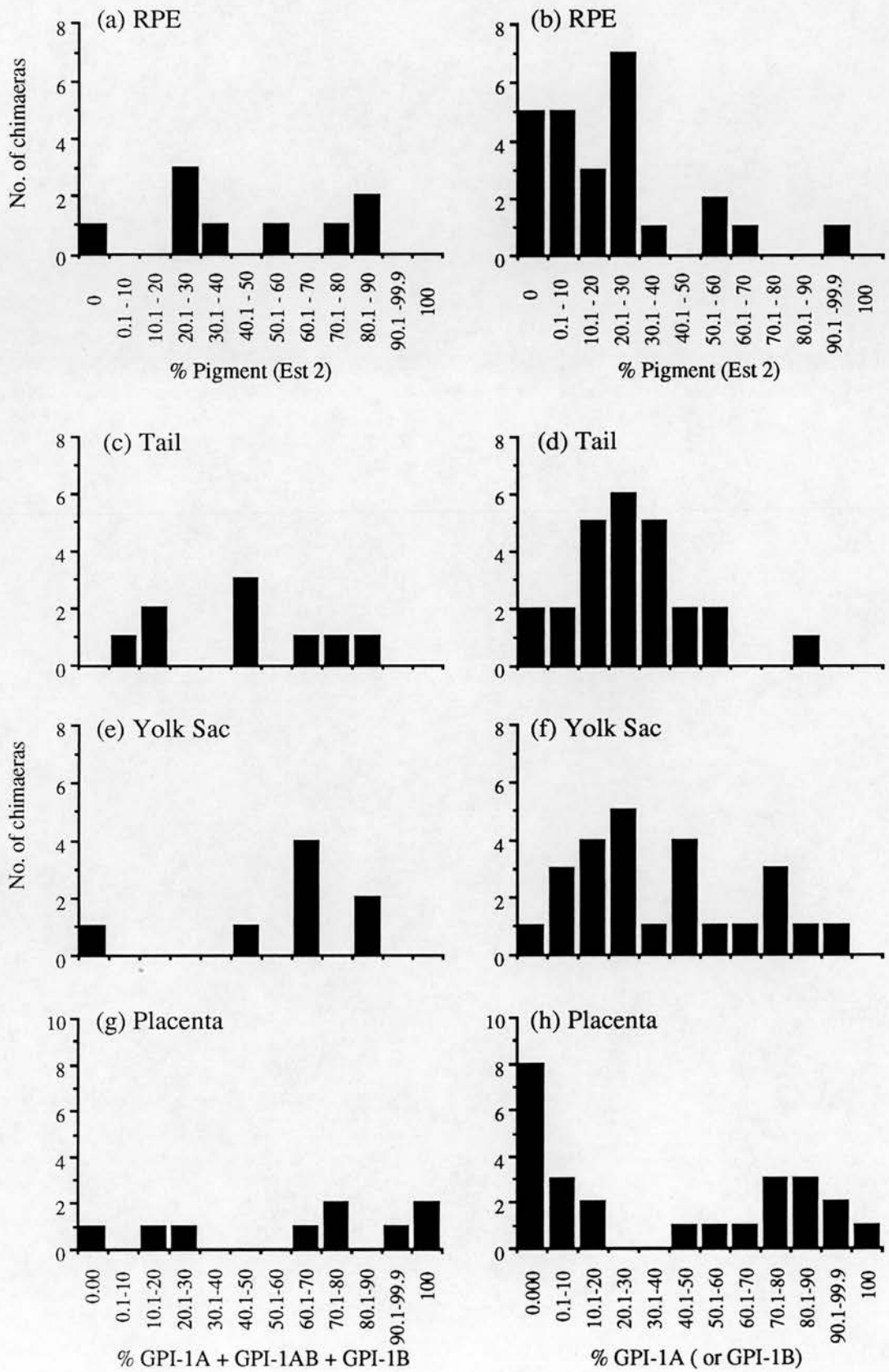
The formula used to calculate the activity of GPI-1A (or GPI-1B) if % GPI-1C observed is 75% the activity of wild type GPI is shown in section 6.2. The formula used to correct the observed % GPI-1A (or GPI-1B) cells in the heterozygous null chimaeras (*a/m* ↔*c/c* and *b/m* ↔*c/c*) is

$$\frac{\%\ \text{GPI-1A Observed} \times 2 \times 100}{(\%\ \text{GPI-1A Observed} \times 2) + (100 - \%\ \text{GPI-1A Observed}) \times 100/75)}$$

This formula takes into account both the reduced activities of GPI-1C (75% of normal) and the expected 50% reduction of GPI-1C activity in heterozygous null *a/m* and *b/m* cells.

Placenta* = Corrected % GPI-1A (or GPI-1AB or GPI-1B) adjusted to allow for 45% of maternal GPI in placenta (James *et al*, 1993). (Formula from footnote Table 2 p 300) where final correction resulted in values of >100% these were taken to be 100%.

Fig 7.1 The distribution of eye pigment and % GPI-1A (+ GPI-1AB + GPI-1B) in tail, yolk sac and placenta of *a/b* ↔*c/c* and *+/m* ↔*c/c* chimaeras
a,c,e & g homozygous normal *a/b* ↔*c/c* chimaeras, N=9.
a) mean = 45.13, c) mean = 43.94, e) mean = 58.73, g) mean = 60.96.
b,d,f, & h heterozygous null *+/m* ↔*c/c* chimaeras, N=25.
b) mean = 21.73, d) mean = 27.15, f) mean = 38.56, h) mean = 36.62.
RPE, retinal pigment epithelium.


(a) RPE
(b) RPE
(c) Tail
(d) Tail
(e) Yolk Sac
(f) Yolk Sac
(g) Placenta
(h) Placenta
No. of chimaeras
No. of chimaeras
0
0.1 - 10
10.1 - 20
20.1 - 30
30.1 - 40
40.1 - 50
50.1 - 60
60.1 - 70
70.1 - 80
80.1 - 90
90.1 -99.9
100
% Pigment (Est 2)
% Pigment (Est 2)
0.00
0.1-10
10.1-20
20.1-30
30.1-40
40.1-50
50.1-60
60.1-70
70.1-80
80.1-90
90.1-99.9
100
% GPI-1A + GPI-1AB + GPI-1B
0.000
0.1-10
10.1-20
20.1-30
30.1-40
40.1-50
50.1-60
60.1-70
70.1-80
80.1-90
90.1-99.9
100
% GPI-1A (or GPI-1B)

chimaeras. Another study from our laboratory has shown a positive correlation between the estimated % pigmented cells in the retinal epithelium and the % GPI-1B in the fetal trunk (where GPI-1B component is pigmented) (West & Flockhart, 1994). In this study only a sample of tail was taken from the fetus, but I wanted to see whether this correlation was still evident, when a smaller fetal sample was taken (Fig 7.3, Table 7.2). In the homozygous normal chimaeras a significantly positive correlation was not apparent between the % eye pigment (Est 2) and the % GPI-1A (+GPI-1AB + GPI-1B) in the tail but r_S was >0.5. The absence of the expected correlations was probably largely a consequence of the small sample size in this group. When Vx15 is removed from this analysis the correlation is still not significantly positve but the r_S value again is >0.5. (Vx15 was a small unpigmented fetus. Its weight was almost two S.D. from the mean (Table 5.5), which suggests it may have been an immature fetus which could account for the lack of eye pigment).

Wilcoxon signed rank tests were used to determine if the contribution of heterozygous normal (*a/b*) cells was significantly different in any of the tissues analysed (Table 7.3). No significant differences were found. When Vx15 was removed there were still no significant differences found.

HETEROZYGOUS NULL CHIMAERAS

Eleven *a/m* ↔ *c/c* chimaeras and 14 *b/m* ↔ *c/c* chimaeras were produced. Firstly, it was necessary to ascertain whether the heterozygous null *a/m* ↔ *c/c* and *b/m* ↔ *c/c* chimaeras were statistically similar enough for them to be grouped together or whether they should be analysed as separate groups. Mann Whitney U-tests were used to determine whether there was a significant difference between the % pigment (Est 2) in the eye, or the % GPI-1A or GPI-1B in the different tissues analysed (tail, yolk sac and placenta) in the *a/m* ↔*c/c* and *b/m* ↔*c/c* chimaeras. The weights were compared using an Unpaired t-test. All these tests showed no significant difference between the two types of heterozygous null chimaeras (Table 7.4), therefore in

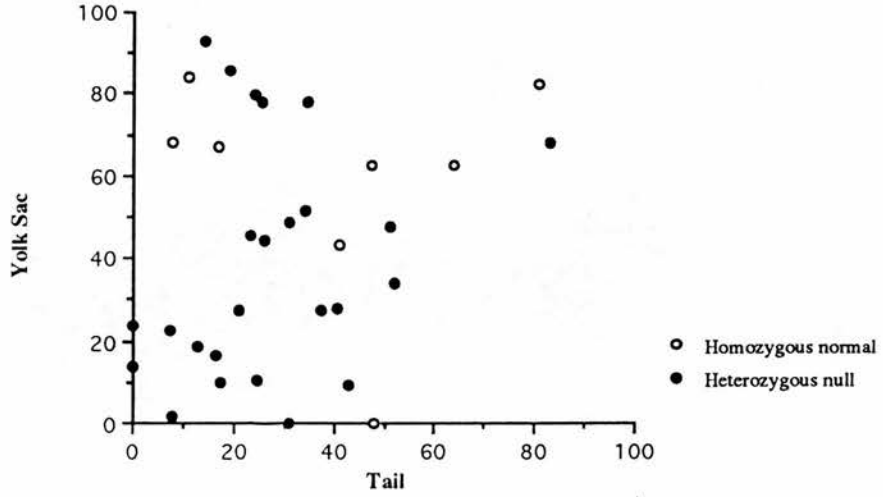
Table 7.2 Spearman rank correlation coefficients, r_S (with P values below) for the % eye pigment and corrected % GPI-1 in the different tissues of *a/b* ↔*c/c* and (*a/m* and *b/m)* ↔*c/c* chimaeras.

	Tail	Yolk Sac	Placenta
a/b ↔*c/c* (N=9)			
Pigment	0.517 P=0.144	0.357 P=0.345	0.228 P=0.416
Pigment (excluding Vx 15)	0.667 P=0.078	0.036 P=0.930	-0.018 P=0.962
Tail		-0.286 P=0.450	0.113 P=0.750
Yolk Sac			0.185 P=0.625
(*a/m* and *b/m)* ↔*c/c* (N=25)			
Pigment	0.629 ***P=0.002***	0.111 P=0.585	0.157 P=0.443
Tail		0.269 P=0.187	0.545 ***P=0.008***
Yolk Sac			0.159 P=0.435

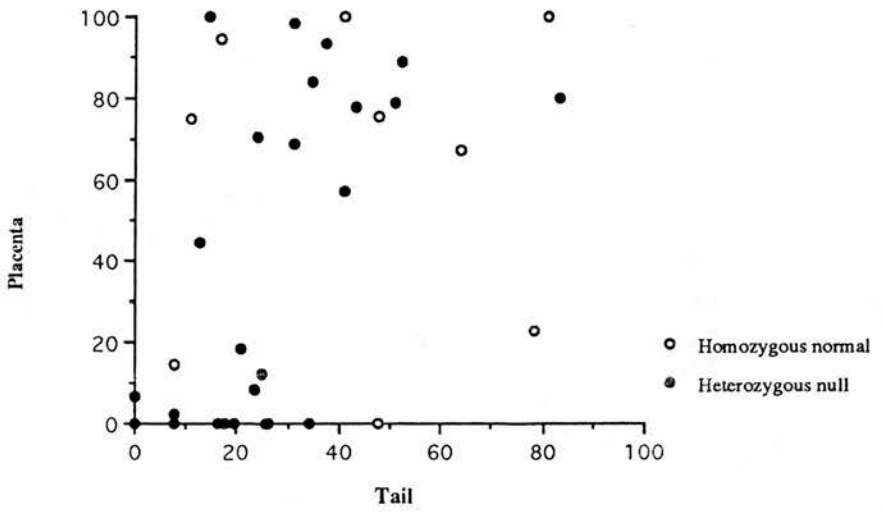
Values are taken to be significant if P<0.05 (in italics)

Figure 7.2 Graph showing the relationship between the % GPI-1A (+ GPI-1AB + GPI-1B) in pairs of tissues of *a/b* ↔*c/c* and (*a/m* and*b/m)* ↔*c/c* chimaeras.

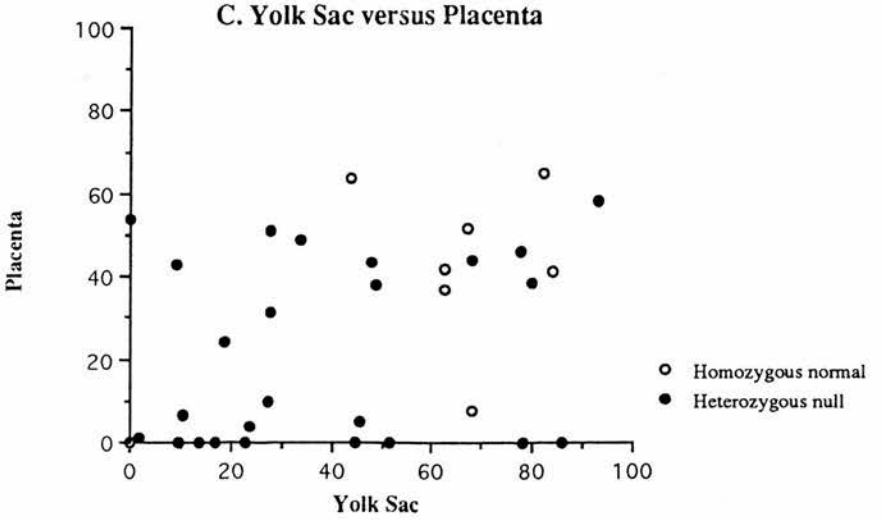
A. Tail versus Yolk Sac



B. Tail versus Placenta



C. Yolk Sac versus Placenta



subsequent analyses the data from these two groups were pooled. The means, standard deviation and standard errors are shown in Table 7.4 which show little variation between the two groups.

The distribution of the eye pigment (Est 2) and the corrected % GPI-1A (+ GPI-1B) in the tail, yolk sac and placenta are shown in Fig 7.1 (b,d,f,h). The distributions for the pigment, tail and yolk sac appeared to be slightly skewed such that most chimaeras contained <50% *+/m* cells. To test whether the visual impression was statistically significant, the proportion of samples with <50% *+/m* contribution was tested against the expected frequency by χ^2 test. For the eye pigment, tail and yolk sac, these proportions (respectively 21:4, 22:3 and 18:7) were significantly different from the expected 1:1 ratio (<50%:>50% *+/m* cells). The χ^2 and P values were respectively 11.56, P<0.001; 14.44, P<0.001; 4.84, P<0.05. The 14:11 ratio (<50%:>50% *+/m* contribution) in the placenta was not significantly different from the expected 1:1 ratio (χ^2=0.36). The skewed distributions raise the possibility that the *+/m* cells may be selected against in the pigmented retinal epithelium, tail and yolk sac.

To test for unusual relationships between tissues Spearman Rank correlations were applied. The relationships in the % GPI-1A (and GPI-1B) between pairs of tissues are shown in Fig 7.2 and Table 7.2. No significantly positive correlation was found between the tail and yolk sac or the yolk sac and placenta but the tail and the placenta were positively correlated (Fig 7.2, Table 7.2). Positive correlations between the % GPI-1A in fetus and placenta have been shown previously in some series of normal ↔ normal chimaeras (West & Flockhart, 1994).

The relationship in the % pigment (Est 2) and corrected % GPI-1A (and GPI-1B) in the tail of the heterozygous null chimaeras is shown in Fig 7.3 and Table 7.2. A significantly positive correlation was observed, which agrees with the previous finding that the proportion of pigment observed in a single mid-section of the eye

correlates well with the proportion of GPI from the pigmented component in the fetal trunk (West & Flockhart, 1994).

Wilcoxon signed rank tests were applied to see if there was any significant difference in the contribution of heterozygous null cells (*+/m*) to the different tissues analysed (Table 7.3). This would give an indication of whether there is tissue-specific selection against *+/m* cells in the tissues analysed. No significant differences were found, although there is a significant skewing towards a low contribution of *+/m* cells in the eye, tail and yolk sac.

HOMOZYGOUS NORMAL CHIMAERAS VERSUS HETEROZYGOUS NULL CHIMAERAS.

Are the heterozygous null cells at a selective disadvantage within aggregation chimaeras compared with normal cells?

The distribution of the eye pigment (Est 2) and the corrected % GPI-1A (+ GPI-1B) in the tail, yolk sac and placenta demonstrated a skewing towards a low contribution of *+/m* cells in the eye, tail and yolk sac whereas the contribution of *a/b* cells in *a/b* ↔ *c/c* chimaeras was fairly evenly balanced. When the ratios (<50%:>50%) from the tissues of these two groups were compared using χ^2 test, there was no significant difference in the eye, tail, or placenta, but the ratios observed in the yolk sac were significantly different (χ^2=5.61; P<0.05).

In order to determine whether there was any difference in the cellular contributions of the *a/m* (and *b/m*) cells to the tail yolk sac and placenta in the heterozygous null chimaeras compared with *a/b* cells (which have no copy of the null allele) in homozygous normal chimaeras, Mann whitney U-tests were applied (Table 7.5). No significant difference was found between *+/m* ↔*c/c* and *a/b* ↔ *c/c* chimaeras in the GPI composition of the tail, yolk sac and placenta. In the retinal pigmented epithelium, the proportion of pigmented cells contributing to the eyes of the

Table 7.3 Probability values from Wilcoxon Signed rank tests comparing the contribution of (GN x NUL) cells in different tissues of *a/b* ↔*c/c* and (*a/m* and *b/m*) ↔*c/c*

		a/b ↔*c/c* N=9	(*a/m* and *b/m*)↔*c/c* N=25
Pigment versus	Tail	0.678	0.107
	Yolk Sac	0.176	0.303
	Placenta	0.327	0.087
Tail versus	Yolk Sac	0.123	0.104
	Placenta	0.173	0.110
Yolk Sac versus	Placenta	0.311	0.989

Table 7.4 Comparison of weights and composition of *a/m ↔c/c* and *b/m ↔c/c* chimaeras* The probability (P) values from Mann Whitney U-tests comparing the % eye pigment observed and % GPI-1A (or GPI-1B) in the tail, yolk sac and placenta. Weights were compared by an unpaired t-test.

	Mean ±S.E.	N	S.D.	t	U	P
% Pigment						
a/m ↔c/c	20.16±5.07	11	16.81	-	73.50	0.848
b/m ↔c/c	22.97±7.47	14	27.94			
% GPI (A or B) Tail						
a/m ↔c/c	21.62±4.68	11	15.53	-	58.00	0.298
b/m ↔c/c	31.50±5.23	14	19.56			
% GPI (A or B) Yolk Sac						
a/m ↔c/c	28.86±8.89	11	29.48	-	44.00	0.071
b/m ↔c/c	46.19±6.79	14	25.42			
% GPI (A or B) Placenta						
a/m ↔c/c	39.99±11.77	11	39.05	-	74.50	0.891
b/m ↔c/c	39.32±11.27	14	42.16			
Weight (g)						
a/m ↔c/c	0.366±0.012	11	0.040	-1.603	-	0.123
b/m ↔c/c	0.335±0.014	14	0.053			

* None of the values are significantly different therefore both types of chimaeras will be pooled.

heterozygous null chimaeras was significantly lower than in the *a/b* ↔ *c/c* chimaeras (Table 7.5). This suggests that *+/m* (*a/m* or *b/m*) cells are at a disadvantage in the eye, or fetus. No such difference was found between the two groups of the fetal tails and we cannot discount the possibility that this result is a chance result of the small number of *a/b* ↔ *c/c* conceptuses. The observed difference between the two groups could also be due to the heterozygous null chimaeras being immature compared with the *a/b* ↔ *c/c* chimaeras, with the eyes being less well developed.

Unpaired t-tests revealed no significant difference in the weights of *+/m* ↔*c/c* and *a/b* ↔ *c/c* chimaeras (Table 7.5). This implies that the heterozygous null chimaeras are not less mature than the homozygous normal chimaeras. This result suggests that the presence of cells with one copy of the null allele of *Gpi-1s* did not affect the growth of chimaeric fetuses. It also suggests that lower contribution of pigmented cells in the eyes of heterozygous null chimaeras is not a consequence of slower development.

TRANSGENIC ANALYSIS

A smaller set of the heterozygous null chimaeras (N=6) were analysed by *in situ* hybridisation to the reiterated β globin transgene. Table A7.1 (see Appendix 7) shows a comparison of the GPI and *in situ* hybridisation estimates of (GN x NUL) cells in the 2 *a/m* ↔*c/c* and 4 *b/m* ↔*c/c* chimaeras that were analysed. A positive correlation was not observed between the GPI data and the transgenic data but the sample size was small.

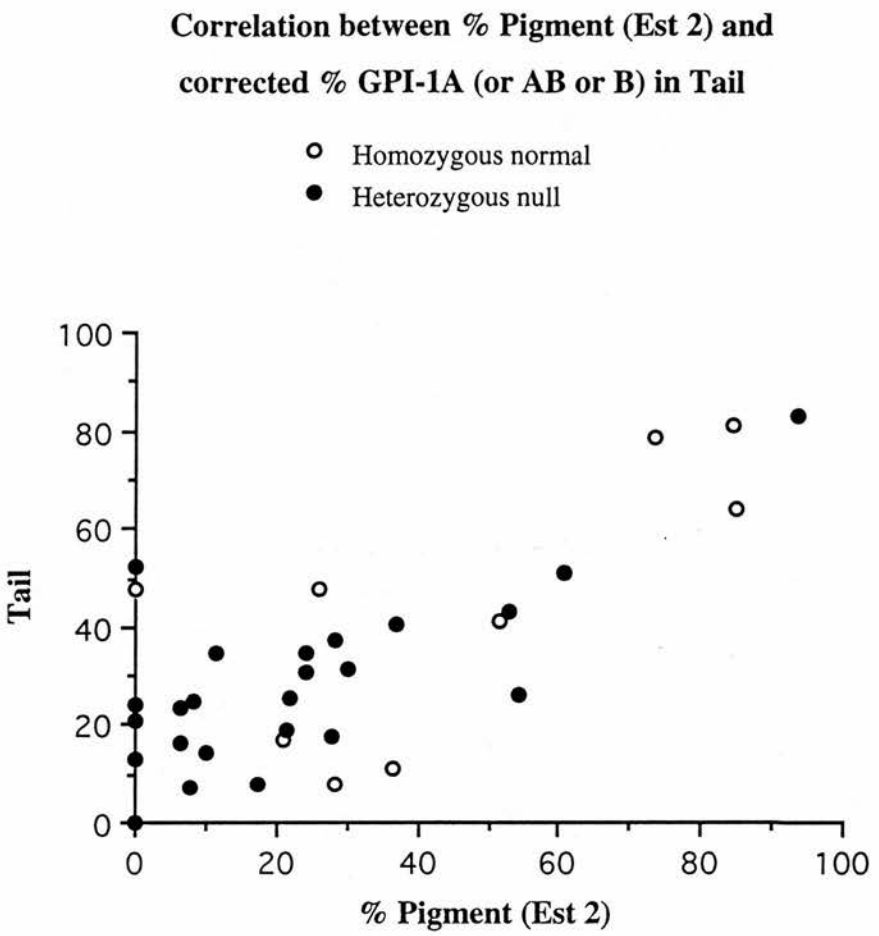


Figure 7.3 Graph showing the relationship between the % Pigment (Est 2) and the % GPI-1A (+ GPI-1AB + GPI-1B) in the tail of homozygous normal (*a/b* ↔*c/c)* and heterozygous null ((*a/m* and*b/m)* ↔*c/c)* chimaeras.

Table 7.5 Comparison of composition (corrected % GPI-1A (+GPI-1AB + GPI-1B) and weights of *a/b* ↔ *c/c* and *+/m* ↔*c/c* chimaeras.
The probability (P) values from Mann Whitney U tests and unpaired t-tests are shown

	Mean ±S.E.	N	S.D.	t	U	P
% Pigment						
(a/m &b/m) ↔*c/c*	21.73±5.4.66	25	23.29	-	60.00	*0.040*
a/b↔*c/c*	45.13±10.09	9	30.26			
% GPI (A or B) Tail						
(a/m &b/m) ↔*c/c*	27.15±3.65	25	18.24	-	73.00	0.123
a/b ↔*c/c*	43.94±9.25	9	27.74			
% GPI (A or B) Yolk Sac						
(a/m &b/m) ↔*c/c*	38.56±5.62	25	28.09	-	62.50	0.115
a/b ↔*c/c*	58.73±9.50	8	28.86			
% GPI (A or B) Placenta						
(a/m &b/m) ↔*c/c*	36.62±8.00	25	39.98	-	75.00	0.143
a/b↔*c/c*	60.96±12.90	9	38.69			
Weight						
(a/m &b/m) ↔*c/c*	0.349±0.010	25	0.050	-0.142	-	0.888
a/b ↔*c/c*	0.346±0.016	9	0.047			

Differences were considered to be statistically significant when P<0.05 (in italics)

7.3 DISCUSSION

After demonstrating no significant difference in composition of *a/m* ↔ *c/c* and *b/m* ↔ *c/c* chimaeras, these two groups of *+/m* ↔ *c/c* chimaeras were compared to the *+/+* ↔ *c/c* chimaeras to test whether the *+/m* cells were at a selective disadvantage in any tissues.

The weights of the homozygous normal and heterozygous null chimaeras were not significantly different, therefore the growth of the heterozygous null chimaeras does not appear to be affected by the presence of cells with one copy of the null allele.

The distribution of the % GPI-1A (+ GPI-1AB + GPI-1B) in the tail, yolk sac or placenta of homozygous normal chimaeras and the % GPI-1A (and GPI-1B) in the tail, yolk sac or placenta of the heterozygous null chimaeras were not significantly different. However the *+/m* ↔ *c/c* chimaeras do look more skewed towards a lower contribution of *+/m* cells in the eye, tail and yolk sac and the contribution of *+/m* cells does differ from the expected 1:1 ratio. This raises the possibility that there is tissue-specific selection against *+/m* cells. However, because of the small numbers of *a/b*↔ *c/c* chimaeras (N=9), it is difficult to make an appropriate comparison and it is still uncertain whether there is selection against *+/m* cells.

The one area where there is some evidence that the heterozygous null cells are selected against is in the pigmented retinal epithelium of the eye. The proportion of pigment observed in the eyes of the heterozygous null chimaeras was significantly smaller than in the eyes of the homozygous normal chimaeras. This may have arisen if heterozygous null cells started with the same capability as normal cells to contribute to the eye but were gradually selected against. It has been demonstrated that parthenogenetic cells become selected against during development in parthenogenetic ↔ fertilized chimaeras. The contribution of parthenogenetic cells to certain tissues (especially the tongue) declines from approximately 13d.p.c. onwards

(Fundele *et al*, 1987). Or it could be that fewer heterozygous null cells are allocated to the retinal pigmented epithelium primordium. The presence of large numbers of heterozygous null cells may be detrimental to the development of the eye. This may also be true of other tissues within the heterozygous null tissues.

This study has shown no significant difference in the contribution of *+/m* cells to the tail, yolk sac and placenta in heterozygous null chimaeras compared with +/+ cells in homozygous normal chimaeras, although a skewing towards a low contribution of +/m cells was apparent in the eye, tail and yolk sac. The pigmented retinal epithelium of the heterozygous null chimaeras contain significantly less pigmented cells than the homozygous normal chimaeras, showing one tissue where there is some evidence that heterozygous null cells are at a selective disadvantage. A further study to produce more homozygous normal chimaeras, may provide more substantial evidence that there is tissue-specific selection against *+/m* cells. Studying different areas of the homozygous normal and heterozygous null chimaeras may identify other tissues where the cells carrying the null allele are selected against.

In the next Chapter the probable and possible homozygous null (*m/m* ↔*c/c*) chimaeras will be analysed to determine whether cells homozygous for the null allele are at a selective disadvantage compared with homozygous normal (*a/b* ↔*c/c*) chimaeras and with heterozygous null (*a/m* ↔*c/c* and *b/m*↔*c/c*) chimaeras.

CHAPTER 8

ANALYSIS OF POSSIBLE AND PROBABLE HOMOZYGOUS NULL FETAL CHIMAERAS

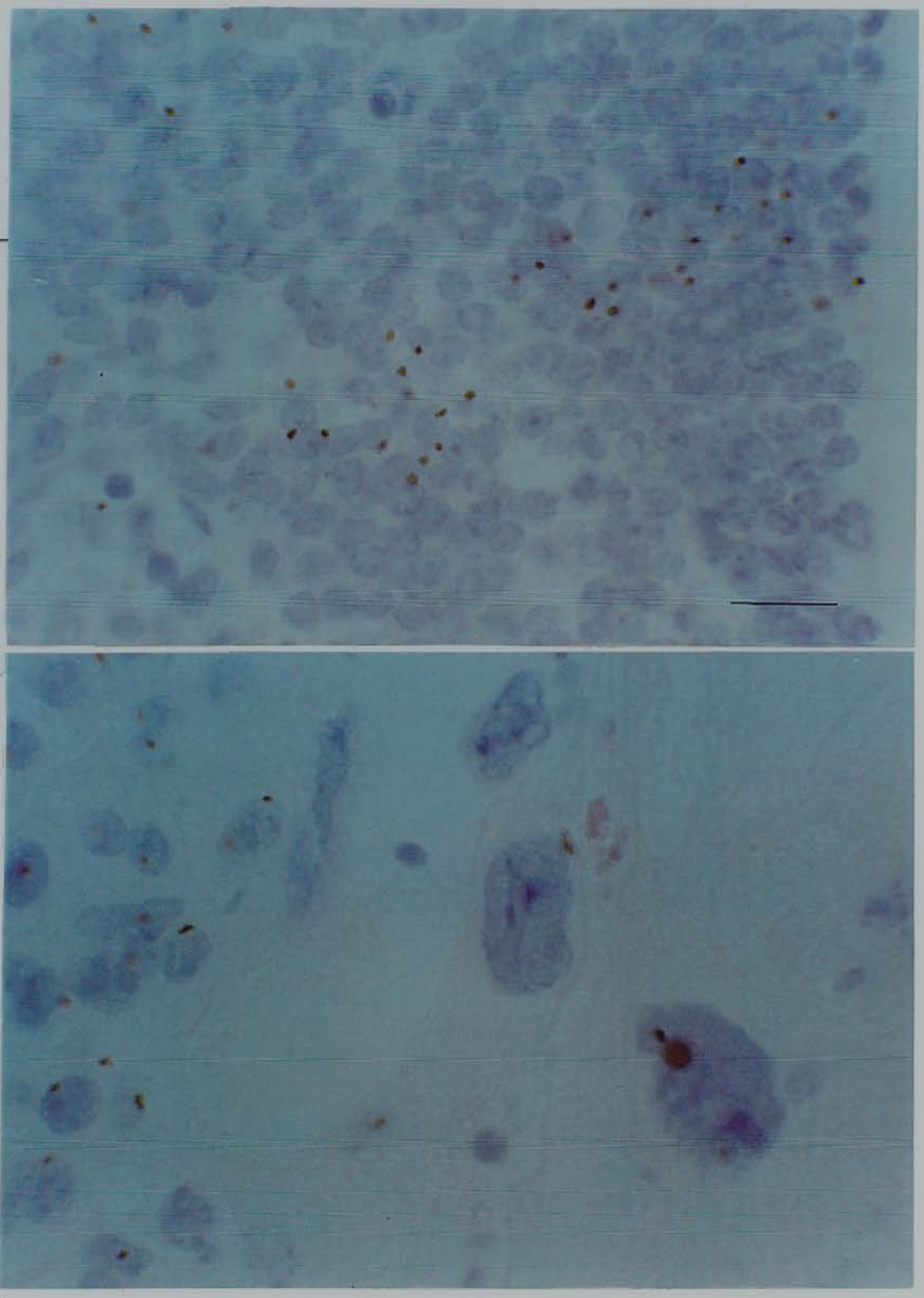
8.1 INTRODUCTION

The previous Chapter has shown that heterozygous null (*a/m or b/m*) cells appear to be at a selective disadvantage in the retinal pigmented epithelium of the eye, and although there was no significant difference in the contribution of *a/m or b/m* cells to the tail, yolk sac and placenta compared to normal cells, the contribution of *+/m* cells to the tail and yolk sac was skewed towards a low contribution. This Chapter will try to determine whether homozygous null (*m/m*) cells are at a selective disadvantage compared to normal cells in aggregation chimaeras.

8.2 RESULTS

The probable homozygous null (*m/m* ↔*c/c*) chimaeras identified in Chapters 5 and 6 were analysed and the probable and possible homozygous null chimaeras were also grouped together to try to determine whether homozygous null cells have a restricted developmental potential. Also 12 chimaeras identified in Chapter 5 as having low % pigment in the eye (Est 1), that is 6 (*b/b* or *b/m*) ↔*c/c* chimaeras from series (i) (see Table 6.2) and 6 (*a/m or b/m*) ↔*c/c* chimaeras from series (ii) (see Table 6.1) were analysed. As the exact genotype of the chimaeras is only known for series (ii), these 12 chimaeras will be referred to as *+/m* ↔*c/c* and +/+ ↔*c/c* chimaeras.

Fig 8.1 Chimaeric tisue sections of A) brain and B) placenta following *in situ* hybridisation to β-globin transgene. 'Spots' in the nucleus identify cells from the GN x NUL component of the chimaera.
Scale bar = 20μm.



(1) IN SITU HYBRIDISATION ANALYSIS OF 3 GROUPS OF CHIMAERAS

For *in situ* hybridisation protocol to β- globin transgene see Chapter 6 and Appendix III(c). Approximately 300 cells were analysed in 6 different tissues of the chimaeras (see Chapter 6, brain, tail, amnion, yolk sac mesoderm, yolk sac endoderm and placenta, Fig 8.1). The putative homozygous null cells were identified in the probable and possible homozygous null chimaeras as pigmented in the eye and by the transgenic marker in other tissues analysed.

The relationships between the % eye pigment (Est 2) and the % transgene detected were tested using Spearman Rank correlations and Wilcoxon sign ranks test (See Appendix 8, Tables A8.1 and A8.2). The statistical analyses were carried out to determine whether there was any significantly positive correlations within or between the cell lineages, and to try to determine whether there was any significant difference in the contribution of transgenic cells to the different lineages which might indicate whether selection against these cells was occuring.

(a) CONTROL +/*m* & +/+ CHIMAERAS (N=12)

Six chimaeras from both series (i) and (ii) with low % eye pigment (Est 1) were analysed by *in situ* hybridisation. A histogram showing the mean % eye pigment (Est 2) and the mean % transgene detected in the six tissues analysed is shown in Fig 8.2A and summary data is tabulated in Table 8.1. As stated previously these chimaeras were chosen because of the low proportion of eye pigment present, therefore the corresponding proportion of transgenic cells in the fetus (brain and tail) should be low. The amnion and yolk sac mesoderm are also primitive ectoderm derivatives so, it would be expected that the proportion of transgenic cells detected in these tissues would also be low. Similar predictions cannot be made for the yolk sac endoderm and the placenta as these tissues are from the primitive endoderm and trophoblast lineages respectively. From the histogram it can be seen that the

Fig 8.2 The mean % (GN x NUL) cells observed in A) (+/+ or +/*m*) ↔ *c/c*, B) probable homozygous null (*m/m* ↔*c/c)* and C) probable and possible homozygous null (*m/m* ↔*c/c)* chimaeras (see Chapter 6).
For each graph pairs of letters denote significant differences e.g. in A) Tail and YS Mes both have the letter (a), the values obtained for these are therefore significantly different.

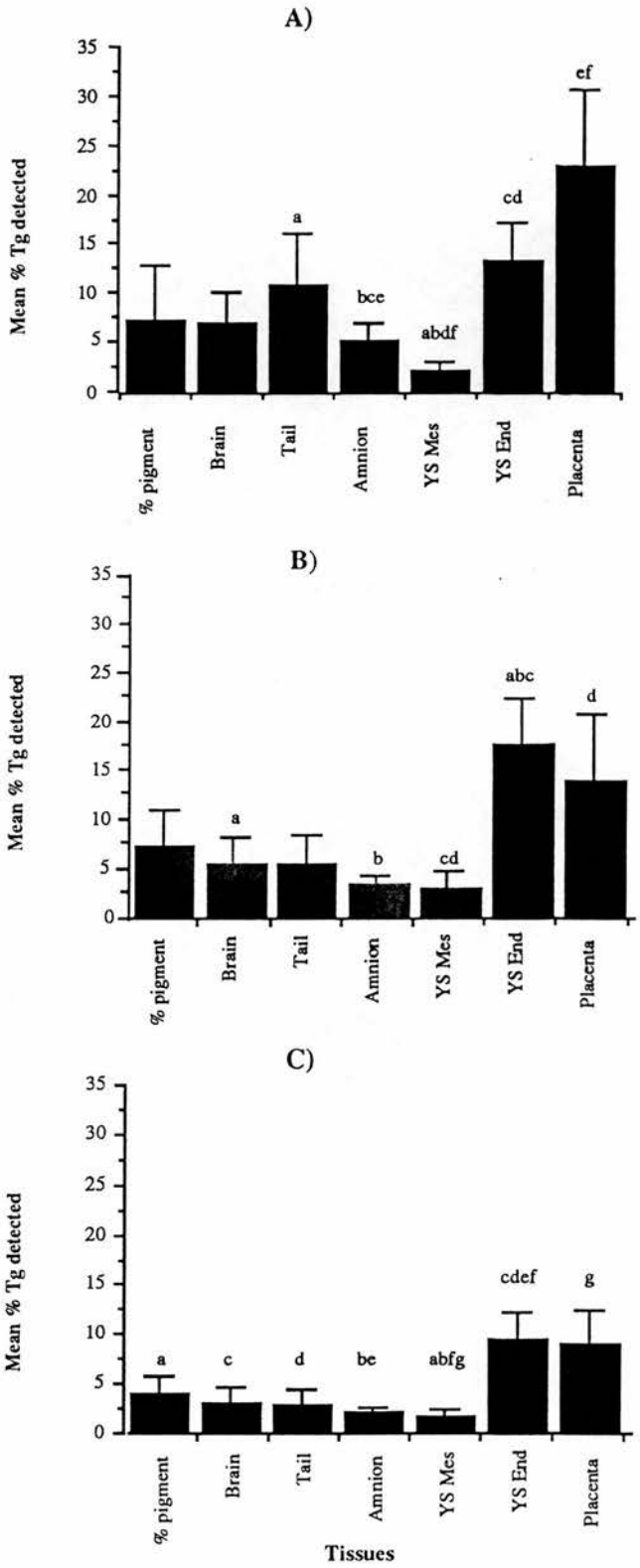

A)
Mean % Tg detected
35
30
25
20
15
10
5
0
a
bce
abdf
cd
ef
% pigment
Brain
Tail
Amnion
YS Mes
YS End
Placenta
B)
Mean % Tg detected
35
30
25
20
15
10
5
0
a
b
cd
abc
d
% pigment
Brain
Tail
Amnion
YS Mes
YS End
Placenta
C)
Mean % Tg detected
35
30
25
20
15
10
5
0
a
c
d
be
abfg
cdef
g
% pigment
Brain
Tail
Amnion
YS Mes
YS End
Placenta
Tissues

Table 8.1 Comparison of mean % Pigment and mean % transgene detected in the tissues of (*+/m or +/+*)↔*c/c*, probable *m/m* ↔*c/c* and probable and possible *m/m* ↔*c/c* chimaeras. The 12 (*+/m or +/+*)↔*c/c* chimaeras analysed were selected for low % eye pigment.

	Mean±S.E	N	S.D.
Pigment			
(*+/m or +/+*)↔*c/c*	7.15±5.69	12*	19.70
probable *m/m* ↔*c/c*	7.12±3.73	14	13.95
probable and possible *m/m* ↔*c/c*	3.84±1.94	28	10.27
Brain			
(*+/m or +/+*)↔*c/c*	6.86±3.21	12	11.11
probable *m/m* ↔*c/c*	5.25±2.73	14	10.23
probable and possible *m/m* ↔*c/c*	3.01±1.47	27	7.65
Tail			
(*+/m or +/+*)↔*c/c*	10.68±5.25	12	18.19
probable *m/m* ↔*c/c*	5.34±2.97	13	10.72
probable and possible *m/m* ↔*c/c*	2.82±1.49	27	7.72
Amnion			
(*+/m or +/+*)↔*c/c*	4.92±2.01	12	6.97
probable *m/m* ↔*c/c*	3.37±1.00	14	3.73
probable and possible *m/m* ↔*c/c*	2.14±0.60	27	3.10
YS mes			
(*+/m or +/+*)↔*c/c*	2.01±0.88	12	3.06
probable *m/m* ↔*c/c*	2.77±1.51	14	5.64
probable and possible *m/m* ↔*c/c*	1.52±0.78	28	4.14
YS end			
(*+/m or +/+*)↔*c/c*	13.18±3.92	12	13.57
probable *m/m* ↔*c/c*	17.28±4.74	14	17.75
probable and possible *m/m* ↔*c/c*	9.31±2.80	28	14.83
Placenta			
(*+/m or +/+*)↔*c/c*	22.30±7.71	12	26.70
probable *m/m* ↔*c/c*	13.69±6.68	14	25.00
probable and possible *m/m* ↔*c/c*	8.83±3.64	28	19.25

*Includes only those analysed by *in situ* hybridisation (selected for low % pigment)

predictions for the primitive ectoderm lineages are realised with the brain, tail, amnion and yolk sac mesoderm having a fairly low contribution of transgenic cells. The contribution of transgenic cells to the yolk sac endoderm and placenta is greater than the tissues of the primitive ectoderm.

Significantly positive correlations were observed between the % eye pigment and the proportion of transgenic cells contributing to the tail and between the tail and amnion, tail and yolk sac mesoderm and amnion and yolk sac mesoderm. Only one significantly positive correlation was observed between the lineages and this was between the brain and yolk sac endoderm (See Appendix 8, Table A8.1). No consistent significant differences were found between the contribution of transgenic cells to the tissues within the primitive ectoderm lineage, or between the primitive endoderm lineage or trophoblast (See Appendix 8, Table A8.2). This analysis revealed no tissue-specific selection against +/+ and *+/m* transgenic cells in control +/+ and *+/m* chimaeras but since the group was a mixture of +/+ ↔ *c/c* and *+/m* ↔ *c/c* chimaeras, the possibility of selection against *+/m* cells cannot be discounted

(b) PROBABLE HOMOZYGOUS NULL CHIMAERAS (N=14)

The data from the probable homozygous null (*m/m* ↔*c/c*) (Table 6.4) chimaeras produced in series (i) (N=6) and (ii) (N=8) were pooled. A histogram of the mean % eye pigment (Est 2) and the mean % transgene detected in the different tissues analysed is shown in Fig 8.2B. The same pattern of low mean % transgene (i.e. % *m/m* cells) detected in the primitive ectoderm lineages and an increase in the primitive endoderm and trophoblast observed in the (*+/m or* +/+) ↔ *c/c* was also apparent in the probable homozygous null chimaeras.

Significantly positive correlations were observed between the tissues analysed within the primitive ectoderm lineage. The placenta correlated significantly with the % eye pigment and the amnion (See Appendix 8). Significant differences were observed in

the contribution of homozygous null transgenic cells to the yolk sac endoderm and to several tissues within the primitive ectoderm lineage, suggesting that there may be selection against homozygous null cells in the primitive ectoderm lineage as compared to the primitive endoderm.

(c) PROBABLE AND POSSIBLE HOMOZYGOUS NULL CHIMAERAS (N=28)

For the following analyses the probable and possible homozygous null chimaeras produced in series (i) and (ii) (Tables 6.3 & 6.4) were pooled to ascertain if including the possible homozygous null chimaeras increased the significance of the statistical tests performed for the probable homozygous null chimaeras. The mean % eye pigment and the mean % transgene detected were plotted as a histogram (Fig 8.1C). Similar to the other two groups of chimaeras, the mean % *m/m* cells detected in tissues within the primitive ectoderm lineages was low and increased in the yolk sac endoderm and placenta.

Very strong significantly positive correlations were observed within tissues of the primitive ectoderm lineage (See Appendix 8). The significantly positive correlations observed between the lineages were weaker than within the primitive ectoderm lineage. Results from the Wilcoxon sign ranks test again suggest that there is selection against homozygous null cells within the primitve ectoderm lineage as compared to the primitive endoderm.

(d) COMPARISON OF IN SITU HYBRIDISATION RESULTS FOR 3 GROUPS OF CHIMAERAS.

Table 8.1 shows the means, standard errors and standard deviations in the % eye pigment and % transgene detected in the three chimaera groups analysed. As stated earlier the (*m/+* and *+/+*) chimaeras were selected for low eye pigment before the transgenic analysis to make this group comparable with the probable and possible homozygous null chimaeras. The histograms of the mean % (GN x NUL) cells

detected in the tissues of the three chimaera groups analysed (Fig 8.1 A-C), show a similar trend, with a low proportion of transgenic cells detected in the tissues of the primitive ectoderm lineage and a higher proportion detected in the tissues of the primitive endoderm and trophoblast lineage. The proportion of pigment and transgenic cells detected in the group of (*+/m or +/+*) ↔*c/c* chimaeras analysed is very similar to the probable homozygous null chimaeras, with the possible plus probable homozygous null chimaeras group being slightly lower. The similarity between the proportion of pigment and transgenic cells detected in these groups of chimaeras may be expected because the (*+/m or +/+*) ↔*c/c* chimaeras were chosen for low eye pigment to correspond to the possible *m/m* ↔*c/c* chimaeras.

(2) COMPARISON OF % EYE PIGMENT AMONG DIFFERENT CLASSES OF CHIMAERAS IN SERIES (ii).

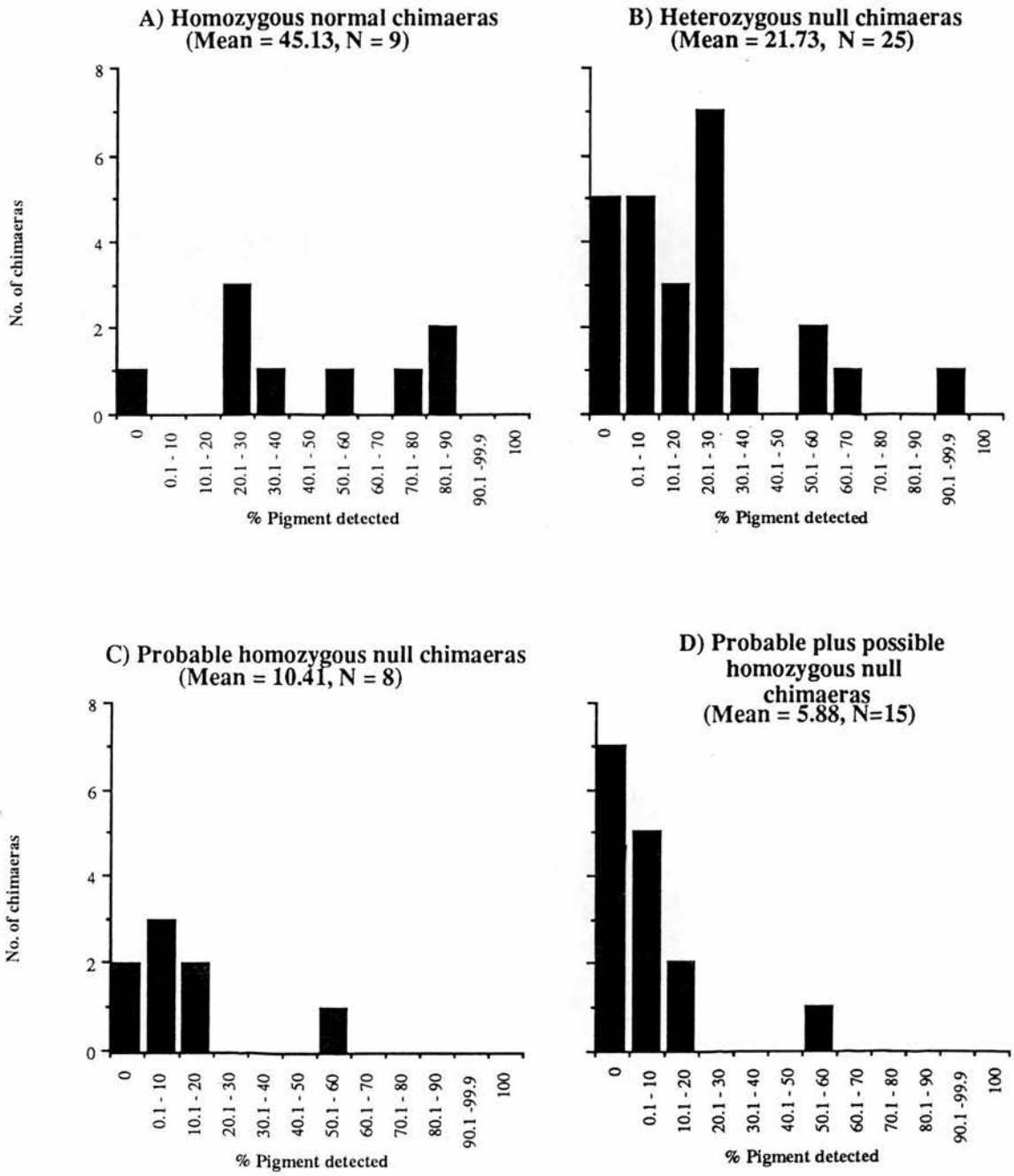
An estimate of the % eye pigment (Est 2) from histological sections was only made on all the chimaeras in series (ii) because the genotype of each chimaera from this group could be identified. In the previous Chapter, the proportion of pigmented cells contributing to the eyes of heterozygous null and homozygous normal chimaeras was compared. The proportion of pigment observed in the retinal pigmented epithelium of the heterozygous null chimaeras was significantly lower than in the homozygous normal chimaeras. The distribution of pigment in the homozygous normal chimaeras was fairly balanced (Fig 8.3A), but the heterozygous null chimaeras were skewed towards a low proportion of eye pigment (Fig 8.3B). The probable homozygous null chimaeras (N=8) also appear skewed towards a low proportion of pigmented cells contributing to the eye (Fig 8.3C). This skewing becomes even more apparent when the possible homozygous null chimaeras are included (Fig 8.3D).

To test whether the visual impression was statistically significant, the proportion of samples with <50% *m/m* contribution was tested against the expected frequency by χ^2 test. The previous Chapter revealed no significant difference from the expected

1:1 ratio (<50%:>50% *a/b* cells) for the homozygous normal chimaeras however the contribution of *+/m* cells in the heterozygous null chimaeras does differ from the expected 1:1 ratio. For the probable homozygous null chimaeras, the observed ratio of 7:1 was significantly different from the expected 1:1 ratio (<50%:>50% *m/m* cells) (χ^2=4.50, P<0.05). When the probable and possible homozygous null chimaeras were pooled, 14:1 again differed significantly from the expected 1:1 ratio (<50%:>50% *m/m* cells) (χ^2=11.27, P<0.001). The skewed distributions for the probable homozygous null (*m/m* ↔ *c/c*) chimaeras and probable plus possible homozygous null chimaeras suggests that the *m/m* cells were selected against in the retinal pigmented epithelium of the eye.

The majority of the probable and possible chimaeras had less than 10% eye pigment detected. Therefore, using 10% as a cut off point, we tested whether there was any significant difference in the proportion of chimaeras that had ≤10%:>10% pigmented cells detected in the eye between the 4 chimaera groups analysed. There was no significant difference in the proportion of homozygous normal (+/+↔ *c/c*) chimaeras with ≤10%:>10% pigment compared to heterozygous null (*m/+* ↔ *c/c*) chimaeras (using Fisher's exact test, 1:7 versus 10:15, P=0.12), or homozygous normal (+/+↔ *c/c*) chimaeras compared to the probable homozygous null (*m/m* ↔ *c/c*) chimaeras (using Fisher's exact test, 1:7 versus 5:3, P=0.06). However when the proportion of homozygous normal (+/+↔ *c/c*) chimaeras with ≤10%:>10% pigment was compared to the probable plus possible homozygous null (*m/m* ↔ *c/c*) chimaeras, a significantly larger proportion of the probable plus possible homozygous null (*m/m* ↔ *c/c*) chimaeras had less than 10% eye pigment detected (using Fisher's exact test, 1:7 versus 12:3, P = 0.003). When the proportion of heterozygous null chimaeras with ≤10%:>10% eye pigment were compared to the possible homozygous null chimaeras, the difference was not significant (10:15 versus 12:3, P = 0.19), but when it was compared to the proportion of probable plus possible homozygous null chimaeras with ≤10%:>10% eye pigment, the difference

Fig 8.3 The distribution of % pigment (Est 2) in homozygous normal (*a/b* ↔ *c/c*), heterozygous null ((*a/m or b/m*) ↔c/c), probable homozygous null (*m/m* ↔*c/c)* and probable and possible homozygous null (*m/m* ↔*c/c)* chimaeras.


A) Homozygous normal chimaeras
(Mean = 45.13, N = 9)
B) Heterozygous null chimaeras
(Mean = 21.73, N = 25)
C) Probable homozygous null chimaeras
(Mean = 10.41, N = 8)
D) Probable plus possible homozygous null chimaeras
(Mean = 5.88, N=15)
No. of chimaeras
% Pigment detected
0
0.1 - 10
10.1 - 20
20.1 - 30
30.1 - 40
40.1 - 50
50.1 - 60
60.1 - 70
70.1 - 80
80.1 - 90
90.1 -99.9
100

became significant, with a greater proportion of probable plus possible homozygous null chimaeras having less than 10% eye pigment present (using Fisher's exact test, 10:15 versus 12:3, P = 0.015).

To test more stringently if the *m/m* cells were being selected against in the eye, Mann Whitney U-tests were applied (Tables 8.2 & 8.3). The proportion of pigmented cells contributing to the eye of probable homozygous null chimareras was significantly less than than homozygous normal (*a/b* ↔*c/c*) chimaeras. This difference was even more significant if the possible homozygous null chimaeras were included. The difference in the % pigment between the homozygous null chimaeras and the heterozygous null chimaeras was less significant and was only statistically significant when the possible homozygous null chimaeras were included with the probables (Tables 8.2 & 8.3).

(3) COMPARISON OF WEIGHTS OF 3 GROUPS OF CHIMAERAS

In series (ii), the weights of the probable homozygous null chimaeras were not significantly different from the weights of the homozygous normal (*a/b* ↔*c/c*) chimaeras, or the heterozygous null ((*a/m or b/m*) ↔ *c/c*) chimaeras (Table 8.2). This suggests that the probable homozygous null chimaeras are not less mature than the homozygous normal or heterozygous null chimaeras, and that having cells with 2 copies of the null allele of *Gpi-1s* did not affect the growth of the conceptuses. Inclusion of the possible homozygous null chimaeras with the probable homozygous null chimaeras, reduced the mean weight slightly. Although this was still not significantly different from the mean weight for the homozygous normal chimaeras, it was significantly different lighter than the heterozygous null chimaeras (Table 8.3).

In series (i), no significant difference was found between the weights of the (*b/m* or *b/b*) ↔*c/c* chimaeras and the probable homozygous null chimaeras. When the

Table 8.2 Comparison of the mean % pigment and mean % weight of chimaeras from series (ii) and (i).

	Mean ±S.E.	N	S.D.
Series (ii)			
% Pigment			
a/b ↔*c/c*	45.13±10.09^{ab}	9	30.26
(*a/m or b/m*)↔*c/c*	21.73±4.66^{c}	25	23.29
Prob *m/m* ↔*c/c*	10.41±6.27^{a}	8	17.72
Prob + poss.*m/m* ↔*c/c*	5.88±3.49^{bc}	15	13.52
Weight (g)			
a/b ↔*c/c*	0.346±0.016	9	0.047
(*a/m or b/m*)↔*c/c*	0.349±0.010^{d}	25	0.001
Prob *m/m* ↔*c/c*	0.311±0.021	8	0.059
Prob + poss.*m/m* ↔*c/c*	0.305±0.014^{d}	15	0.053
Series (i)			
Weight			
(*b/m or b/b*) ↔*c/c*	0.330±0.006	45	0.040
Prob *m/m* ↔*c/c*	0.320±0.010	6	0.025
Prob and poss *m/m* ↔*c/c*	0.309±0.008	13	0.030

Pairs of letters in superscript denote significant differences between groups e.g. the % pigment detected in *a/b* ↔*c/c* chimaeras is significantly different from the pigment detected in Prob *m/m* ↔*c/c* chimaeras (both with a letter a). For probability values see Table 8.3

Table 8.3. The probability (P) values from Mann Whitney U tests comparing the % eye pigment observed and unpaired t-test comparing the weight in chimaeras from series (ii) and series (i).

	Eye pigment (U-values)	Weight (t-values)
Series (ii)		
a/b ↔*c/c* versus probable *m/m* ↔*c/c*	12.00	1.348
	P=*0.0210*	P=0.198
a/b ↔*c/c* versus probable and possible	16.5	1.906
m/m ↔*c/c*	P=*0.0024*	P=0.0700
(*a/m* and *b/m*) ↔*c/c* versus probable	69.00	1.779
m/m ↔*c/c*	P=0.193	P=0.085
(*a/m* and *b/m*) ↔*c/c* versus probable	96.00	2.632
and possible*m/m* ↔*c/c*	P=*0.011*	P=*0.0122*
Series (i)		
(*b/m* and *b/b*) ↔*c/c* versus probable		0.604
m/m ↔*c/c*	ND	P=0.549
(*b/m* and *b/b*) ↔*c/c* versus probable		1.781
and possible*m/m* ↔*c/c*	ND	P=0.080

Difference in % eye pigment observed and weight were considered to be statistically significant when P<0.05 (in italics)

possibles were included the difference between the weights was also non-significant (Table 8.2 & Table 8.3). Again, this suggests that the probable (and probable plus possible homozygous null chimaeras) are not less mature than (*b/b or b/m*) ↔ *c/c* chimaeras

8.3 DISCUSSION

A comparison of the proportion of homozygous null cells contributing to different tissues within the probable, and probable plus possible homozygous null chimaeras and *+/m* and +/+ cells contributing to tissues of the (*+/m or* +/+) ↔*c/c* chimaeras was made. The transgenic data was tested to determine whether there were correlations within the primitive ectoderm lineage and also between lineages of the three groups of chimaeras. From previous studies (West *et al*, 1984, James *et al*, 1993, and West & Flockhart, 1994, West *et al*, 1995), it would be expected that the tissues within the primitive ectoderm lineage would be positively correlated. Failure to find the expected correlations might indicate a tissue-specific selection against homozygous null cells.

In the (*+/m or* +/+) ↔ *c/c* chimaeras some of the tissues within the primitive ectoderm lineage did not correlate significantly. However, Wilcoxon signed rank tests revealed no consistent difference in contribution. The contribution to the yolk sac mesoderm was low but it was also the lowest for the other groups analysed. The failure to find the expected correlations could be accounted for by the narrow range of contribution of *+/m* and +/+ cells in this group of chimaeras.

In the probable homozygous null chimaeras and the probable plus possible homozygous null chimaeras significantly positive correlations were observed between the tissues of the primitive ectoderm lineages. No significant difference in the contribution of *m/m* cells to these tissues were observed either, suggesting that *m/m* cells were under similar selection pressure (if any) in all tissues within this

lineage. The similarity between the composition of all of the primitive ectoderm derivatives implies that *m/m* cells are not selected against more stringently in the fetus than all of the extraembryonic tissues.

In probable homozygous null (*m/m* ↔*c/c*) chimaeras and probable plus possible homozygous null chimaeras, there was a significantly higher contribution of *m/m* cells to the yolk sac endoderm compared to some of the tissues within the primitive ectoderm lineage. This suggests that there may be selection against *m/m* cells within the primitive ectoderm lineage compared with the primitive endoderm lineage. The placenta had a significantly higher contribution of *m/m* cells than the yolk sac mesoderm (in the probable nulls) but the evidence for selection against *m/m* cells within the trophoblast lineage is not as strong because the yolk sac mesoderm had the lowest contribution of transgenic cells in all the groups analysed.

Several predictions could have been made about the survival of homozygous null cells in chimaeras from the morphological analysis of homozygous (*m/m*) null embryos. The extraembryonic endoderm survives fairly well in these mutant embryos and therefore in chimaeras there may have been a greater contribution of these cells to this tissue. From the *in situ* analysis of the homozygous null chimaeras there does appear to be selection against *m/m* cells within tissues of the primitive ectoderm lineage compared to the yolk sac endoderm. Mesoderm is very sparse in the homozygous null embryos, although this is most likely due to there being insufficient energy available to fuel gastrulation, *m/m* cells may make a much lower contribution to mesoderm derived tissues in homozygous null chimaeras, such as the amnion and yolk sac mesoderm. The contribution of *m/m* cells to the non-mesoderm derived tissues within the primitive ectoderm lineage was not significantly greater to the mesoderm derived tissues within the primitive ectoderm lineage.

The proportion of eye pigment detected in the eyes of the probable homozygous null chimaeras (and also the probable plus possible homozygous null chimaeras) was

significantly less than in the homozygous normal chimaeras. The distribution of % eye pigment in the probable homozygous null chimaeras also supports this, with distribution being skewed towards a low contribution of pigmented cells. The probable homozygous null chimaeras did not have significantly less pigment than the heterozygous null chimaeras, but the difference was significant when the possible homozygous null chimaeras were included with the probable *m/m* ↔*c/c* chimaeras. The results from testing whether a significantly higher proportion of the probable (and probable plus possible homozygous null chimaeras) had ≤10% eye pigment detected compared to the homozygous normal chimaeras and heterozygous null chimaeras revealed that when the probable homozygous null chimaeras were considered without the possible homozygous null chimaeras, there was no significant difference, but when the probable and possible homozygous null chimaeras were pooled, a significantly higher proportion of these chimaeras had ≤10% eye pigment detected. This suggests, that if all of the possible homozygous null chimaeras are genuine homozygous null chimaeras, then that as for the heterozygous null chimaeras, the homozygous null (*m/m*) cells are at a disadvantage in the retinal pigmented epithelium and may be at a greater disadvantage than *+/m* cells.

The cells of the retina obtain energy via aerobic glycolysis (Warburg, cited in Krebs, 1981). If these cells cannot obtain other substrates to fuel glycolysis, they would either proliferate more slowly or might die because of their lack of GPI. Therefore the presence of large numbers of these cells in the eye may be detrimental to its development. The homozygous null cells may initially have had the same potential as homozygous normal cells to contribute to the eye but were gradually selected against as development progressed. This type of selection has been demonstrated in parthenogenetic ↔ fertilized chimaeras, where parthenogenetic cells are selected against in certain tissues as development proceeds (Fundele *et al*, 1987). Alternatively, it may be that homozygous null cells were always at a disadvantage compared to normal cells and that only a small proportion were allocated to the

retinal primordium. However, this would not explain why in one *m/m* ↔*c/c* chimaera the % pigment detected was >50% (Vx51). This chimaera may have escaped the selection process, or it may be that there is a threshold level, that the homozygous null cells cannot exceed.

This Chapter has compared the contribution of transgenic cells in different tissues of probable, probable plus possible homozygous null chimaeras and (*+/m or +/+*) ↔*c/c* chimaeras. Comparing the proportion of transgenic cells contributing to different primitve ectoderm tissues between the chimaera groups was not feasible because there was insufficient time to undertake *in situ* hybridisation on every chimaera. Instead the (*+/m or +/+*) ↔*c/c* chimaeras with low % eye pigment were selected for analysis to correspond to the probable homozygous null chimaera group. However the mean proportion of *m/m* cells was low in each primitive ectoderm tissue studied and overall the *m/m* ↔ *c/c* chimaeras resembled the +/+ ↔ *c/c* chimaeras selected for a low +/+ contribution in the primitive ectoderm lineage.

The contribution of homozygous null (*m/m*) cells to the yolk sac endoderm (primitive endoderm lineage) was significantly higher than in some tissues of the primitive ectoderm suggesting that there is more stringent selection against *m/m* cells in the primitive ectoderm lineage of probable (and probable plus possible) homozygous null chimaeras. Thus, rather than selection confined to the fetus or tissue specific selection it may be lineage specific selection that occurs. There is also selection against *m/m* cells in the pigmented retinal epithelium of probable (and probable plus possible) homozygous null chimaeras compared with normal cells.

If time had permitted, a more thorough investigation, comparing the proportion of probable homozygous null cells in probable homozygous null chimaeras with *a/b* cells in homozygous normal chimaeras, and heterozygous null cells in (*a/m or b/m*) ↔ *c/c* chimaeras would have been carried out. This would give a better indication of whether the homozygous null cells are at a selective disadvantage within the

primitive ectoderm lineage compared to the primitive endoderm and trophoblast lineages.

CHAPTER 9

SPATIAL ANALYSIS OF PIGMENT DISTRIBUTION IN FETAL CHIMAERA EYES

9.1 INTRODUCTION

Chapters 7 and 8 have demonstrated the contribution of *+/m* cells and *m/m* cells to the pigmented retinal epithelium was significantly lower in heterozygous null and homozygous null chimaeras respectively, compared to normal cells in homozygous normal chimaeras. The aim of this Chapter was to test whether homozygous null (*m/m*) cells, and heterozygous (*+/m*) null cells can mix to the same extent as homozygous normal (+/+) cells in homozygous null, heterozygous null and homozygous normal chimaeras respectively.

One possibility is that homozygous *m/m* cells are only able to survive and proliferate if they are in contact with wildtype +/+ cells. If so, there would be no large patches of m/m cells; they would either be broken up into smaller patches or they would not be able to expand beyond a size where all the *m/m* cells were in contact with a +/+ cell. (In this Chapter, a "patch" refers to a contiguous group of cells of like genotype). If proliferation of patches of *m/m* cells was inhibited beyond a certain size, this would also account for the low proportion of *m/m* cells found in *m/m* ↔ +/+ chimaeras. If there were no large patches of *m/m* cells, the mean size of patches of pigmented cells in pigmented *m/m* ↔ albino +/+ chimaeras would be smaller than in pigmented +/+ ↔ albino +/+ chimaeras with a similar proportion of pigmented cells.

If the pigmented epithelium primordium of an *m/m* ↔ +/+ chimaera initially had a moderately high proportion of *m/m* cells (say 50%), patches of *m/m* cells would

probably be widely distributed within the retinal pigmented epithelium. If the proliferation of these patches was subsequently impaired and they did not break up into smaller patches the proportion would decline (say 10%) but the patches would remain widely distributed. If so, it might be expected that the distribution of pigmented patches in pigmented, *m/m* ↔ albino +/+ chimaeras would be more widespread than in pigmented +/+ ↔ albino +/+ chimaeras with a similar proportion of pigmented cells because in pigmented +/+ ↔albino +/+ chimaeras with low proportions of pigmented retinal epithelium cells, the patches tend to be clustered rather than distributed throughout the tissue (see West, 1978).

The aim of this Chapter is to test whether *m/m* and +/+ cells mix normally in the fetal retinal pigmented epithelium of pigmented *m/m* ↔ albino +/+ chimaeras. In particular to test two specific predictions. (1) The mean length of pigmented patches (after correction for the effect of variation in % pigmented cells) would be lower in *m/m* ↔ +/+ chimaeras than in +/+ ↔ +/+ chimaeras. (2) The pigmented patches would be more widely dispersed over the retinal pigmented epithelium in *m/m* ↔ +/+ chimaeras than in +/+ ↔ +/+ chimaeras with a similar proportion of pigmented cells.

Two types of analysis were performed, a) a one dimensional analysis, determining whether the size of pigmented patches in the different genotypes of chimaeras produced in series (ii) were significantly different and b) a two dimensional analysis visually determining whether the distribution of pigmented patches were different in the heterozygous null and homozygous null chimaeras.

9.2 MATERIALS AND METHODS

Analysis of % eye pigment (Est. 2) from histological sections was performed in collaboration with Mr. B. A. Hodson, Department of Obstetrics and Gynaecology, University of Edinburgh.

a) One dimensional analysis

The lengths (in mm) of pigmented and unpigmented patches were measured around the circumference of the eye starting at the choroid fissure. The mean length of a pigmented patch of cells and the proportion of pigmented patches of cells in the whole eye could be calculated from this. The formula of Roach (1969) 1/1-p (see Appendix 9), which calculates the expected length of a patch of cells, if the cells are randomly arranged in a linear string was used as a correction factor to correct for the variation of pigmented patch length that is caused by the proportion of pigment present. This correction factor was applied to the mean patch length to calculate the mean corrected patch length (West, 1976).

b) Two dimensional analysis

Serial reconstructions of the pigmented retinal epithelium of 4 chimaeric eyes were produced. These were 2 heterozygous null chimaeras (Vx29 & Vx4) and 2 homozygous null chimaeras (Vx26 & Vx37). This was done by measuring the amount of pigmented to non-pigmented cells in serial sections of the eye, starting at the mid-section and building a composite picture of a series of sections. This analysis was a visual comparison only and the distributions of patches were not compared statistically.

9.3 RESULTS

a) One dimensional analysis

Table 9.1 compares the mean corrected patch sizes in the homozygous normal (*a/b* ↔ *c/c*), heterozygous null ((*a/m or b/m*) ↔ *c/c*), probable homozygous null (*m/m* ↔*c/c*) and probable plus possible homozygous null (*m/m*↔*c/c*) chimaeras. The mean corrected patch size was slightly larger in the *a/b* ↔ *c/c* and (*a/m or b/m*) ↔

Table 9.1 Comparison of the mean corrected patch lengths (mm) in the *a/b* ↔ *c/c*, (*a/m or b/m*) ↔ *c/c* , probable *m/m*↔ *c/c* and probable plus possible *m/m* ↔ *c/c* chimaeras in series (ii).

Chimaera	No.	Mean± S.E	S.D.
a/b ↔ *c/c*	8	11.86±0.84	2.43
(*a/m or b/m*) ↔ *c/c*	21	11.39±0.50	2.31
probable *m/m* ↔ *c/c*	6	10.15±1.20	2.95
probable plus possible *m/m* ↔ *c/c*	8	10.97±1.06	3.00

Relationship between mean corrected patch size and % pigmented cells in the RPE of 12.5d fetal chimaeras

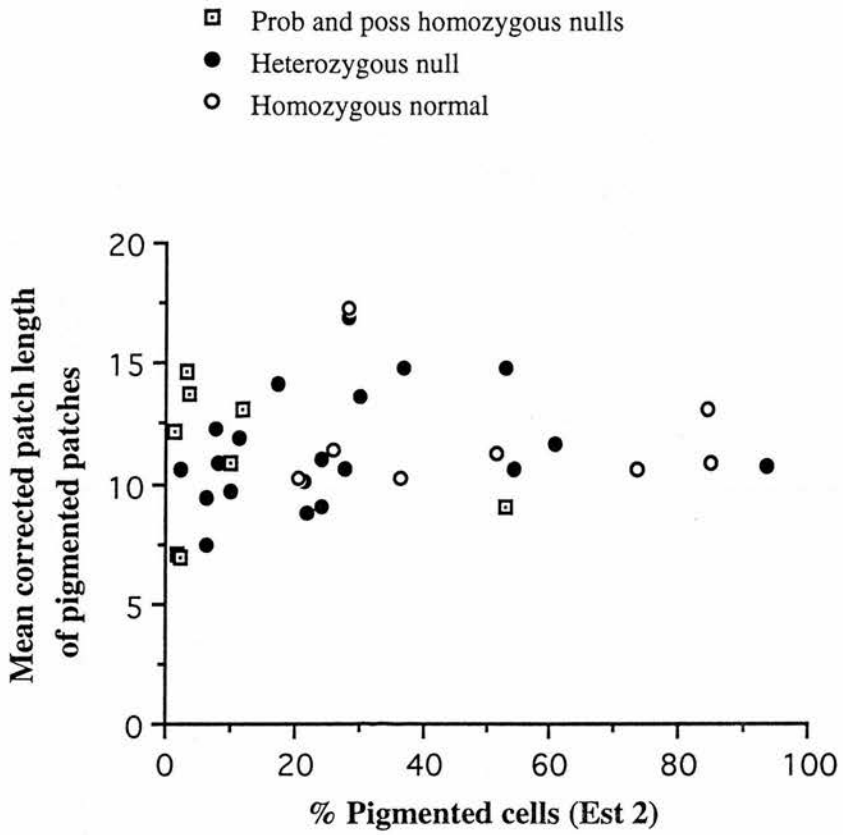


Fig 9.1 Graph of the % eye pigment (Est 2) plotted against the mean corrected patch length (mm).

c/c chimaera groups, although when unpaired t-tests were applied there was no significant difference between any of the groups analysed.

This similarity in mean corrected patch size is also illustrated in Fig 9.1. The % eye pigment (Est 2) in histological sections was plotted against the mean corrected patch length (mm) for the homozygous normal, heterozygous null and probable plus possible homozygous null chimaeras. The mean corrected patch length for the homozygous normal chimaeras fell roughly on a straight line, with the heterozygous null and probable plus possible homozygous null chimaeras falling slightly above and below this line.

b) Two dimensional analysis

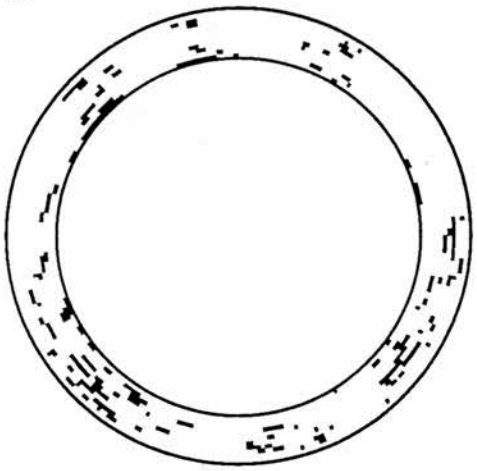
Serial reconstructions of the pigmented retinal epithelium of two probable homozygous null chimaeras which had approximately 10% pigment were made (Vx 37 and Vx 26). Since no homozygous normal chimaeras (*a/b* ↔ *c/c*) had equivalent amounts of eye pigment present, these were compared to two heterozygous null chimaeras with similar proportions of eye pigment present. The distribution pattern of the pigmented cells in the pigmented retinal epithelium of these four chimaeras are shown in Fig 9.2 and appear very similar in the 4 chimaeras suggesting that homozygous (*m/m*) null cells are not more scattered than the heterozygous null (*+/m*) cells.

9.4 DISCUSSION

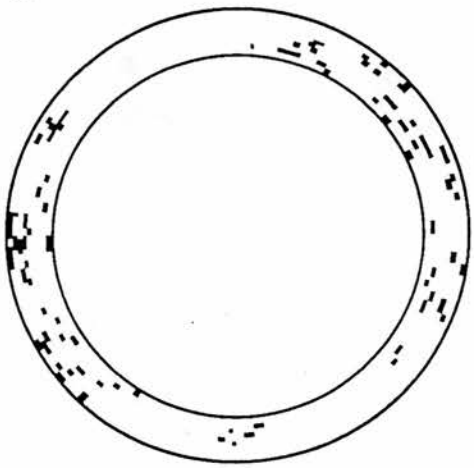
Chapters 7 and 8 demonstrated that the heterozygous null (*+/m*) and homozygous null (*m/m*) pigmented cells appeared to be selected against in the pigmented retinal epithelium of the eye compared with homozygous normal (*a/b*) cells. However when the mean corrected patch size was compared, there was no significant difference between any of the groups analysed. The serial reconstructions of the two homozygous null chimaeras and two heterozygous null chimaeras with

Fig 9.2 Serial reconstructions of eye pigment distributions in 2 heterozygous null and 2 homozygous null chimaeras. A) Vx29, heterozygous null (*a/m* ↔ *c/c*) chimaera, with 11.4% pigment, B) Vx4, heterozygous null (*b/m* ↔ *c/c*) chimaera, with 10.2% pigment, C) Vx26, homozygous null (*m/m* ↔ *c/c*) chimaera with 12.4% pigment and D) Vx37, homozygous null (*m/m* ↔ *c/c*) chimaera with 10.3% pigment.

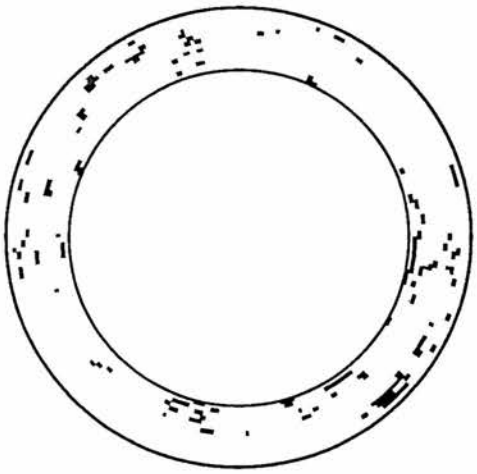
A



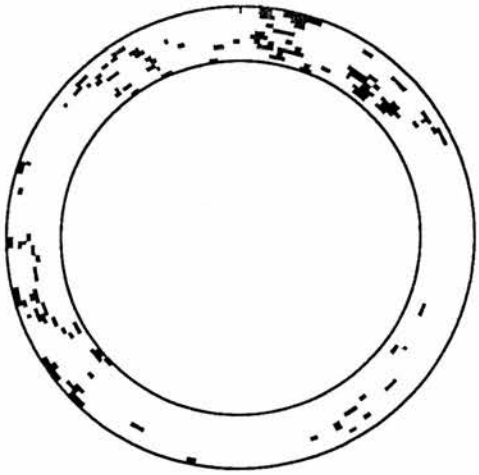
C



B



D



approximately 10% pigment demonstrated visually that the distribution of pigment in these chimaeras was very similar. Both sets of observations suggest that cell mixing was occuring to the same extent in *m/m* ↔+/+ and either +/*m* ↔ +/+ or +/+ ↔ *c/c* chimaeras

Even if patches of m/m cells in *m/m* ↔+/+ chimaeras tend to be smaller than normal, there are two reasons why this may not be apparent from the analysis of the retinal pigmented epithelium of 12.5 day *m/m* ↔ +/+ chimaeras. First, the pigmented retinal epithelium is a single layer of cells. The homozygous null cells may be able to obtain nutrients from normal wild type neighbouring cells from above in the neural retina and below in the mesenchyme. If transfer of nutrients occurs then this may explain why the mean corrected patch size does not differ between the three chimaera groups. If the size of the mean corrected patch length in the homozygous null chimaeras had been significantly smaller than in homozygous normal chimaeras, it may have been due to *m/m* cells at the edge of the patches being rescued by contact of wild type cells, and *m/m* cells in the middle of patches (surrounded by other *m/m* cells) dying because they received no nutrients .The size of *m/m* patches would as a result be smaller.

Second, the similarity of the mean corrected patch size in the chimaera groups analysed could be due to the stage of analysis. In control 12.5 day chimaeras the mean corrected patch size is already small measuring only slightly larger than the size of one cell (Table 9.1 and West ,1976). It would therefore be difficult to demonstrate a significant reduction in patch size at this stage of development. A further study investigating the patch size in adult chimaera eyes, once the pigmented retinal epithelium had differentiated completely, would give a better indication of whether there was a significant reduction in patch size in homozygous null or heterozygous null chimaeras.

CHAPTER 10

CAN HOMOZYGOUS GPI NULL CHIMAERAS SURVIVE TO TERM?

10.1 INTRODUCTION

Chapters 5 & 6 have shown that homozygous (*m/m*) and heterozygous GPI (*a/m* and *b/m*) null cells are able to survive until at least 12.5 days when combined with normal cells in aggregation chimaeras. In order to ascertain whether these homozygous GPI null cells can survive longer, three embryo tranfers from series (ii) were allowed to develop to term to try to address this question. Chapters 7 and 8 have shown that in at least one tissue (the retinal pigmented epithelium) there appears to be selection against heterozygous and homozygous null cells. This selection against these cells may continue during development and could result in the absence of heterozygous and homozygous null cells in certain tissues of adult chimaeras, or complete absence. In previous studies where abnormal $\leftrightarrow$ normal chimaeras are produced, the potential of the abnormal cells to contribute to certain tissues becomes restricted during development to term (Fundele et al, 1990).

Homozygous null chimaeras were identified initially by coat colour chimaerism and by GPI type, they were then test mated to identify the genotype of the offspring and verify their chimaeric status.

10.2 MATERIALS AND METHODS

CHIMAERA PRODUCTION AND IDENTIFICATION

Chimaeras were produced as described in Chapter 5 and Appendix VI, VII and VII.

Series (ii) experimental plan (taken from Chapter 5.2)

[GN (*b/m*) female x NUL (*a/m*) male↔ CF1(*c/c*) x CF1 (*c/c*)] to produce offspring of the GPI types *a/m*↔ *c/c*, *a/b*↔ *c/c* , *b/m*↔ *c/c* and *m/m*↔ *c/c*. Fig 5.4 shows a gel electrophoresis plate showing the bands expected from chimaeric offspring from this experiment.

IDENTIFICATION OF CHIMAERAS

Chimaeras were identified by coat colour and by GPI type. Blood samples were taken from the chimaeras at 5 weeks old (*in situ* hybridisation protocol in Appendix III(d)). Approximately 10ml blood was taken from the caudal vein of the anaesthetized chimaeras and diluted in 0.1ml 3.2% tri sodium citrate for GPI electrophoresis

BREEDING

Adult chimaeras were mated to CF1 (*c/c*) albino mice and offspring typed by coat colour and electrophoresis as above. First generation offspring (from the probable homozygous null chimaera) were subsequently mated to pigmented BF1 (*b/b*) mice to check the genotype of offspring.

DISSECTION OF ADULT CHIMAERAS

After several litters had been produced from the adult chimaeras they were sacrificed by CO_2 gas. The eyes were removed, fixed and processed for histology. A range of tissues were dissected out and cut in half. One part was taken for GPI electrophoresis

assay and placed in an eppendorf with 100ml H_2O. The remainder was processed for *in situ* hybridisation analysis except in the case of the ovary and oviduct where the right was processed for *in situ* hybridisation and the left assayed for the presence of GPI. The different male and female reproductive organs were also taken. The small intestine was cut into 3 parts and designated as I, II and III (I being closest to the stomach, II the middle and III closest to the caecum and appendix), each of which were then halved and processed as above.

GPI ELECTROPHORESIS.

Adult tissue samples for gel electrophoresis were diluted 1: 5 in H_2O and run as in Chapter 5.2.

HISTOLOGY

The tissues were fixed and processed for histology as in Chapter 5.2 with the exception of the pituitary which was fixed for only 3hrs before removing to 70% alcohol.

Chimaera AK2 died while giving birth; the tissues were not dissected out immediately as in the other chimaeras, therefore the tissues were not as fresh when fixed and processed for histology.

10.3 RESULTS

Eleven mice were produced from the three transfers that were allowed to develop to term. The GPI phenotypes and various proportions of pigment detected in the coat of the term chimaeras are shown in Table 10.1. Homozygous GPI null chimaeras would be expected to produce GPI-1C only in the blood sample and have a variegated coat.

Table 10.1 The approximate % pigment detected in the coat and GPI phenotype of each chimaera.

Chimaera No.	Coat (Est % pig)	GPI phenotype	Interpretation
AK10 (F)	0	C	non-chimaeric
AK11 (M)	0	C	non-chimaeric
AK5 (M)	70	A + C	*a/m* ↔ *c/c*
AK9 (F)	40	B + C	*b/m* ↔ *c/c*
AK8 (F)	45	B + C	*b/m* ↔ *c/c*
AK3 (F)	70	B + C	*b/m* ↔ *c/c*
AK4 (M)	80	AB + C	*a/b*↔ *c/c*
AK7 (F)	90	AB + C	*a/b*↔ *c/c*
AK6 (M)	95	AB + C	*a/b*↔ *c/c*
AK1 (F)	<1	C	chimaeric, possible *m/m* ↔ *c/c*
AK2 (F)*	10	C	*m/m* ↔ *c/c*

(F) = female
(M) = male
* = putative homozygous null chimaera

Two of the mice produced were entirely albino (AK11 and AK10) and therefore probably non-chimaeric. Unfortunately AK11 died before breeding data could be obtained. One had a tiny spot of colour on it's nose (AK1) which eventually faded.

The remaining 8 mice had clearly variegated coats, 7 were between 45 and 95% pigmented and 1 mouse was approximately 10% pigmented (Fig 10.1A & B and Table 10.1). Initial GPI analysis was performed on blood samples at 5 weeks. Only GPI-1C was produced by chimaeras AK1, AK10 and AK 11. Three of these gave GPI-1B + GPI-1C bands, one produced GPI-1A + GPI-1C and 3 were GPI-1AB + GPI-1C. Approximately 10% pigment was detected in the coat of AK2 and this female produced GPI-1-C only (AK2, Fig 10.1B). AK2 was therefore classified as a probable homozygous GPI null chimaera.

BREEDING

All the chimaeras were mated to albino GPI-1C (*c/c*), CF1 mice. If a chimaera was of the *a/m*↔ *c/c* type , three types of offspring would be expected. The *a/m* germ line would produce pigmented offspring that would be either *a/c* (GPI-1AC) or *m/c* (GPI-1C) and the *c/c* germ line would produce *c/c* (GPI-1C) albino offspring (Table 10.2). If a chimaera was of the *b/m* ↔ *c/c* type, offspring would be either GPI-1BC or GPI-1C only. If a chimaera was of the *a/b* ↔ *c/c* type, offspring would be GPI-1AC, GPI-1BC or GPI-1C only. Offspring from homozygous GPI null chimaeras (*m/m* ↔ *c/c*) would all be GPI-1C only but could be pigmented or albino. Non-chimaeric (*c/c)* mice would produce albino GPI-1C offspring (Table 10.2)

Offspring of the chimaeras were analysed to identify whether viable gametes were produced in each germline. If a male chimaera produces offspring from only one type of germ cell, it could be that the chimaera is XX ↔ XY with only XY sperm being produced, or it may be an XY ↔ XY chimaera with one genotype being excluded from the germ line. Female chimaeras (XX ↔ XX), will have chimaeric

Fig 10.1(A) A litter of chimaeric pups at two weeks of age.

(B) Putative homozygous null chimaera

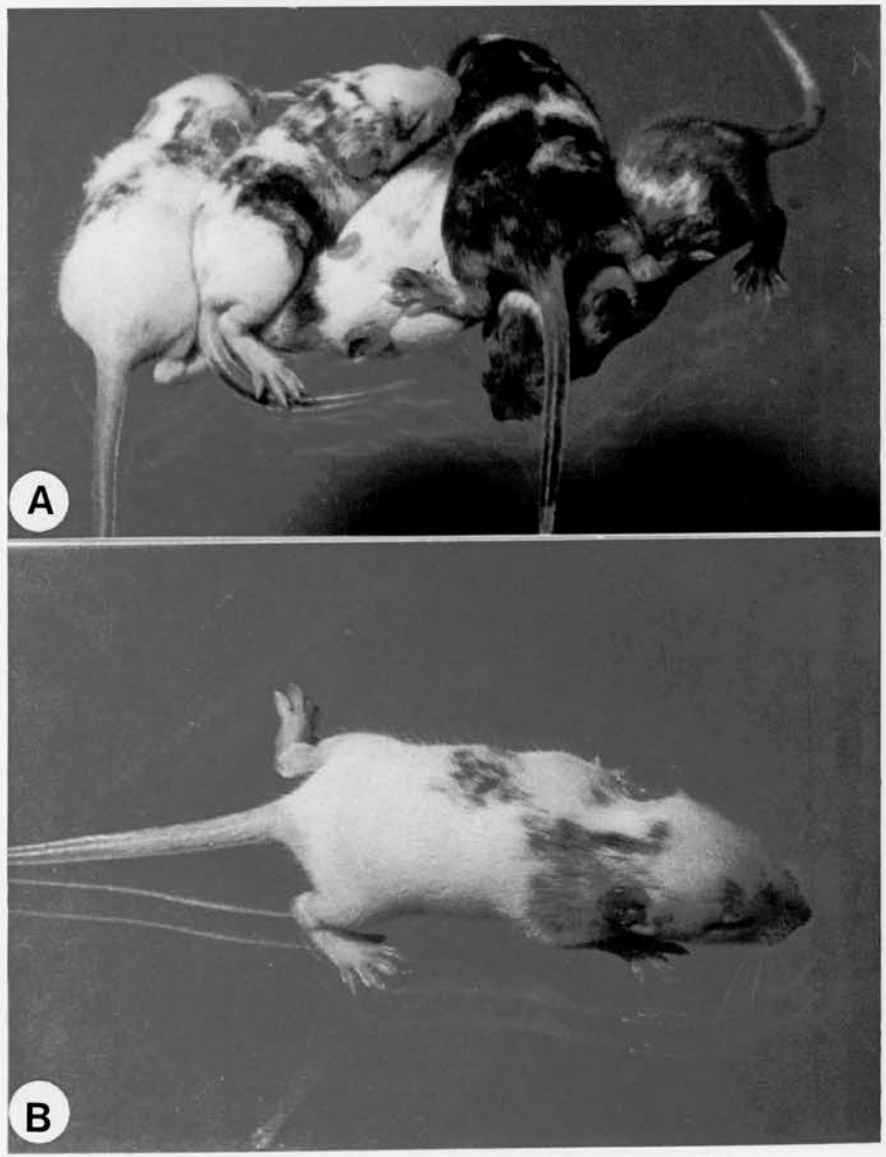

A
B

Table 10.2 The expected GPI genotypes and coat colour of offspring from chimaeric individuals mated to an albino *c/c* strain (if chimaera is XX ↔ XX or XY ↔ XY).

Mating	Offspring	
	Pigmented	Albino
(*a/m*↔*c/c*) x *c/c*	*a/c*	*c/c*
	m/c	
(*b/m* ↔*c/c*) x *c/c*	*b/c*	*c/c*
	m/c	
(*a/b* ↔ *c/c*) x *c/c*	*a/c*	*c/c*
	b/c	
(*m/m* ↔ *c/c*) x *c/c*	*m/c*	*c/c*

ovaries and will produce offspring from both germ cell types unless one genotype component is predominant. Few XX ↔ XY chimaeras would be expected to develop as females unless few XY cells contributed to the gonads. In such cases, XY germ cells would not be expected to produce mature oocytes.

Table 10.2 shows the expected offspring and Table 10.3 the observed offspring that were produced from mating the adult chimaeras to albino CF1 mice. AK1 and AK10 produced only albino GPI-1C offspring. AK10 was therefore non-chimaeric. AK1 had a small patch of colour on it's nose (<1% pigmented) which faded as it matured. This mouse was therefore chimaeric, but the exact genotype could not be determined from the GPI analysis of the 5 week old blood sample because only GPI-1C was produced (presumably because the GN x NUL component was absent from the blood or below the limit of detection). This chimaera could therefore be a possible homozygous null chimaera. All of the offspring were albino, GPI-1C which also suggests that only the *c/c* cell population contributed to the germ line.

The 3 GPI-1B + GPI-1C chimaeras (AK3, AK8 and AK 9, Table 10.3) were all female. From these 3 chimaeras, both pigmented and albino GPI-1C offspring were produced, but only pigmented GPI-1BC offspring. The phenotype of these chimaeras and the offspring they produced implies that they are XX ↔ XX chimaeras. AK8 and AK9 produced a proportion of pigmented offspring that roughly corresponded to the proportion of pigment detected in the coat. AK3 produced a slightly lower proportion of pigmented offspring compared to the proportion of pigment detected in the coat, this may reflect a smaller proportion of *b/m* germ cells present in the ovary compared with *c/c* germ cells.

The one GPI-1A + GPI-1C chimaera (AK5) produced offspring which were GPI-1AC and pigmented, and both albino and pigmented GPI-1C only. The majority of the offspring from this chimaera were produced from the *a/m* germ cells, and suggests that in this chimaera there may be a greater proportion of *a/m* germ cells

Table 10.3 Breeding data. The GPI genotype of each of the offspring and coat colour are shown. The chimaeras were mated to homozygous *Gpi-1s*^{c}, *c/Gpi-1s*^{c}*c* mice.

Chimaera	Sex	No. offspring	GPI phenotype of pigmented offspring			GPI phenotype of albino offspring			% pigmented offspring	Mean % pigment in coat & eye* (somatic)		
			AC	BC	C	AC	BC	C		Coat	Eye	% Tg cells in blood
non-chimaeric *c/c*												
AK10	F	13	0	0	0	0	0	13	0	0	0	ND
b/m ↔*c/c*												
AK3	F	46	0	8	4	0	0	35	26	70	65	ND
AK8	F	33	0	5	4	0	0	24	27	45	45	ND
AK9	F	29	0	6	12	0	0	11	62	40	20	ND
a/m ↔*c/c*												
AK5	M	66	28	0	30	0	0	8	88	70	ND	43
a/b ↔*c/c*												
AK4	M	56	0	0	0	0	0	56	0	80	ND	90
AK6	M	65	39	26	0	0	0	0	100	95	ND	27
AK7	F	54	19	21	0	0	0	14	74	90	45	45
possible *m/m*↔ *c/c*												
AK1	F	48	0	0	0	0	0	48	0	<1	<1	ND
m/m ↔*c/c*												
AK2	F	28	0	0	8	0	0	20	29	10	15*	8

* estimates of eye pigment are a mean of left and right eye (pigmented epithelium plus the choroid).
ND = not done
* see Fig 10.3.

compared to *c/c*. One of the GPI-1AB + GPI-1C chimaeras (AK4) produced offspring all of which were albino and GPI-1C only. This chimaera was a male and only produced offspring from the GPI-1C component, which suggests this was likely to be an XX ↔ XY chimaera (particularly since the coat was 80% pigmented). The *a/b* component of the chimaera was likely to be XX and therefore unable to produce male germ cells.

Chimaera AK6 is also an GPI-1AB + GPI-1C male chimaera. Pigmented GPI-1AC and GPI-1BC offspring were produced from this mating but no GPI-1C offspring were produced. This could again suggest that this chimaera was an XY ↔ XX, with the *a/b* component in this chimaera being XY. However since the coat was 95% pigmented, this chimaera could be XY ↔ XY with the germ line predominantly or exclusively derived from the *a/b* genotype. The final chimaera of this type was a female (AK7) and produced both pigmented GPI-1AC and GPI-1BC offspring as well as GPI-1C albino offspring. This implies that this chimaera was an XX ↔ XX chimaera, because offspring from each germ line were produced. Further investigation of the ovaries and testis of these chimaeras could be carried out by *in situ* hybridisation to a Y probe to determine the proportion of XY cells present within these tissues (and other tissues) within the chimaeras. This would also give an indication of whether the identification of these chimaeras as either XX ↔ XX, XX ↔ XY or XY ↔ XY is correct.

AK2, was a putative homozygous GPI null chimaera. In order to ascertain whether this chimaera was fertile or if the abnormal genotype could be transmitted through the germ line, it was crossed to an albino CF1 male. The types of offspring expected when a homozygous GPI null chimaera is mated to an albino, GPI-1C strain (CF1) are shown in Table 10.2. If offspring were produced from this mating they should be either pigmented *m/c* or albino *c/c* . Both of these GPI types would be visualized as a C band on an electrophoresis plate. However if this female was one of the other

chimaera types, different genotypes would be observed among the pigmented offspring. Twenty eight offspring were produced, 20 of which were albino and GPI-1C and 8 which were pigmented and GPI-1C. This provides further evidence that the chimaeric female is a homozygous GPI null chimaera. If AK2 was, for example, really *a/m* ↔ *c/c* 50% of the offspring would be expected to be GPI-1AC (*a/c*) rather than GPI-1C. The chance of getting 8 pigmented offspring which were all GPI-1C is low [$(^1/_2)^8$ or P=0.004]. This is further evidence that AK2 was really an *m/m* ↔ *c/c* chimaera.

From the first generation offspring, 5 pigmented (3 males, 2 females) and 4 albino (2 males and 2 females) were test-mated to pigmented, BF1 (*b/b*) to verify the genotype (Table 10.4). If the female was in fact a *m/m* ↔ *c/c* chimaera, then all of the pigmented offspring from the first generation matings should be *m/c* and all of the albino offspring should be *c/c*. Both genotypes would be phenotypically GPI-1C but the test-mating would distinguish between them. Both *b/m* (GPI-1B) and *b/c* (GPI-1BC would be produced from *m/c* x *b/b* test-matings whereas only *b/c* (GPI-1BC) would be produced from *c/c* x *b/b* test-matings. The test matings confirmed that each of the pigmented animals tested was an *m/c* heterozygote, as expected if AK2 was an *m/m* ↔ *c/c* chimaera.

ELECTROPHORESIS OF ADULT TISSUES

GPI electrophoresis was performed on the various tissues dissected from the adult chimaeras (Table 10.5), to try to determine (1) whether the mice that appeared to be non-chimaeric in the coat and blood had a low level of GPI-1A (or GPI-1AB or GPI-1B) in other tissues, (2) whether AK2 (the putative homozygous null chimaera) had a low level of GPI-1A (or GPI-1AB or GPI-1B) in other tissues, (3) if AK1, which had a small patch of colour on it's nose, was a possible *m/m* ↔ *c/c* chimaera and (4) if all of the tissues within the chimaeras were in fact chimaeric or if one cell population was excluded in some tissues.

Table 10.4 Offspring from matings between 9 progeny of chimaera AK2 (see Table 10.3) and BF1 strain (*b/b*) mice.

Mating	Total no. offspring	GPI phenotype of offspring B	BC
Pigmented *m/c* progeny x *b/b*			
1	46	23	23
2	37	18	19
3	18	8	10
4	21	10	11
5	10	7	3
Total	132	66	66
Albino *c/c* progeny x *b/b*			
1	32		32
2	24		24
3	21		21
4	13		13
Total	90		90

Table 10. 5 Genotypes of tissues removed from adult chimaeras
A. Female chimaeras

		AK3	AK9	AK8	AK7	AK11	AK1	AK2
Ectodermal derivative								
Brain		B+C	B+C	B+C	AB+C	C	C	C
Mesodermal derivatives								
Blood		B+C	B+C	B+C	AB+C	C	C	C
Heart		B+C	B+C	B+C	AB+C	C	C	C
R. Ovary and Oviduct (in situ)								
L. Ovary and Oviduct (GPI)		B+C	B+C	B+C	AB+C	C	C	C
Uterine Horn (both R and L)		B+C	B+C	B+C	AB+C	C	C	C
Kidney (both R and L)		B+C	B+C	B+C	AB+C	C	C	C
R. Hind Limb Muscle		BC	BC	BC	BC	C	C	C
L. Hind Limb Muscle		BC	BC	BC	BC	C	C	C
Mammary Gland		B+C	B+C	B+C	AB+C	C	C	C
Endodermal derivatives								
Tongue		BC	BC	BC	AB+C	C	C	C
Liver	Posterior lobe							
	L. Lateral lobe	B+C	B+C	B+C	AB+C	C	C	C
	Medial lobe	B+C	B+C	B+C	AB+C	C	C	C
	R. Lateral lobe	B+C	B+C	B+C	AB+C	C	C	C
Pancreas		B+C	B+C	B+C	AB+C	C	C	C
Spleen		B+C	B+C	B+C	AB+C	C	C	C
Lung		B+C	B+C	B+C	AB+C	C	C	C
Thymus		B+C	B+C	B+C	AB+C	C	C	C
Small Intestine	I	B+C	B+C	B+C	AB+C	C	C	C
	II	B+C	B+C	B+C	AB+C	C	C	C
	II	B+C	B+C	B+C	AB+C	C	C	C
Caecum and Appendix		B+C	B+C	B+C	AB+C	C	C	C
Large Intestine		B+C	B+C	B+C	AB+C	C	C	C
Bladder		B+C	B+C	B+C	AB+C	C	C	C

R = Right, L= Left
B + C = GPI-1B + C, BC + GPI-1B + GPI-1BC + GPI-1C
AB+C = GPI-1AB + C, ABC = GPI-1A + GPI-1AB + GPI-1B/GPI-1AC + GPI-1BC +GPI-1C.
C = GPI-1C

Table 10.5 continued
B. Male chimaeras

		AK11	AK4	AK6	AK5
Ectodermal derivatives					
Brain		C	AB+C	AB+C	A+C
Mesodermal derivatives					
Blood		C	AB+C	AB+C	A+C
Heart		C	AB+C	AB+C	A+C
Seminal Vesicles (both R and L)*		ND	AB+C	AB+C	A+C
Testis (both R and L)		C	AB+C	AB+C	A+C
Epididymus (both R and L)		ND	AB+C	AB+C	A+C
Kidney (both R and L)		ND	AB+C	AB+C	A+C
R. Hind Limb Muscle		ND	ABC	ABC	AC
L. Hind Limb Muscle		C	ABC	ABC	AC
Endodermal derivatives					
Tongue		C	ABC	ABC	AC
Liver	Posterior lobe	ND	AB+C	AB+C	A+C
	L. Lateral lobe	C	AB+C	AB+C	A+C
	Medial lobe	ND	AB+C	AB+C	A+C
	R. Lateral lobe	C	AB+C	AB+C	A+C
Pancreas		ND	AB+C	AB+C	A+C
Spleen		C	AB+C	AB+C	A+C
Lung		C	AB+C	AB+C	A+C
Thymus		C	AB+C	AB+C	A+C
Small Intestine	I	ND	AB+C	AB+C	A+C
	II	ND	AB+C	AB+C	A+C
	III	ND	AB+C	AB+C	A+C
Caecum and Appendix		ND	AB+C	AB+C	A+C
Large Intestine		ND	AB+C	AB+C	A+C
Bladder		C	AB+C	AB+C	A+C

R= Right, L=Left
A + C = GPI-1A + GPI-1C, AC = GPI-1A + GPI-1AC + GPI-1C
B + C = GPI-1B + C, BC + GPI-1B + GPI-1BC + GPI-1C
AB+C = GPI-1AB + C, ABC = GPI-1A + GPI-1AB + GPI-1B/GPI-1AC + GPI-1BC +GPI-1C.
C = GPI-1C

In the 2 albino chimaeras, GPI-1C was produced in all the tissues analysed (a smaller range of tissues were dissected from the albino male that died before breeding data was collected), consistent with the conclusion that these animals were non chimaeric. All of the tissues analysed from AK1 produced GPI-1C only. The other component of this chimaera either made such a small contribution to the tissues analysed that the electrophoresis system could not detect it or else it was excluded from all of these tissues. The expected genotypes were produced from the tissues analysed from the remaining chimaeras, confirming the blood genotype (Table 10.6).

The tongue and limb muscles are syncytial. In the mixed genotype chimaeras (AK3, 4, 5, 6, 8 & 9) the GPI results from these tissues showed that both types of cells had contributed to the syncytium. This means that in the case of GPI-1A + C chimaeras, the tongue and limb muscles produced GPI-1AC (Table 10.6, Fig 10.2). In one GPI-1AB + C chimaera (AK 7) the hind limb muscles produced only the GPI-1BC phenotype (3 bands AC/B + BC + C, Fig 10.2) rather than the GPI-1ABC phenotype (5 bands, A + AB + AC/B + BC + C, Fig 10.2) as was observed in the hind limb muscles of the other chimaeras of this type. This was probably due to the muscle being composed of cells which were predominantly *Gpi-1s*$^{c/c}$ which would produce an excess of GPI-1C monomers. If only a small proportion of the cells were *Gpi-1s*$^{a/b}$ then only a small proportion of GPI-1A and GPI-1B monomers would be produced and the GPI-1C monomers would compete with these to form dimers. Nearly all the GPI-1A and GPI-1B monomers would dimerise with the GPI-1C monomers, rather then with each other. The GPI-1AC band comigrates with the GPI-1B band on an electrophoresis plate. The same competition for the GPI-1B monomers would take place and most would dimerize with the excess GPI-1C monomers rather than form homodimers. This could explain the electrophoresis result from the limb muscles in this chimaera. So the GPI-1BC phenotype that was observed on the electrophoresis plate could in fact be a GPI-1AC, GPI-1BC and

Table 10.6 In situ hybridisation analysis of tissues from AK2 (homozygous null chimaera)

Tissue	Transgenic signal
Ectodermal derivatives	
Brain	+
Mesoderm derivatives	
Blood	+
Heart	-
L ovary and oviduct	+
R.Kidney	-
Uterus	+
Endodermal derivatives	
Liver L. Lateral	+
Pancreas	-
Spleen	-
Lung	-
Thymus	+
Small Intestine I	+
Large Intestine	-
Caecum and Appendix	-
Bladder	+

+ = transgenic cells detected
- = no transgenic cells detected

Fig 10.2 Electrophoresis plates after staining for GPI in different tissues from chimaeric mice.

A) *b/m* ↔ *c/c* chimaera, AK8

B) *a/b* ↔ *c/c* chimaera, AK4

C) *a/m* ↔ *c/c* chimaera, AK5

D) *a/b* ↔ *c/c* chimaera, AK7

Circles denote hind limb muscles.

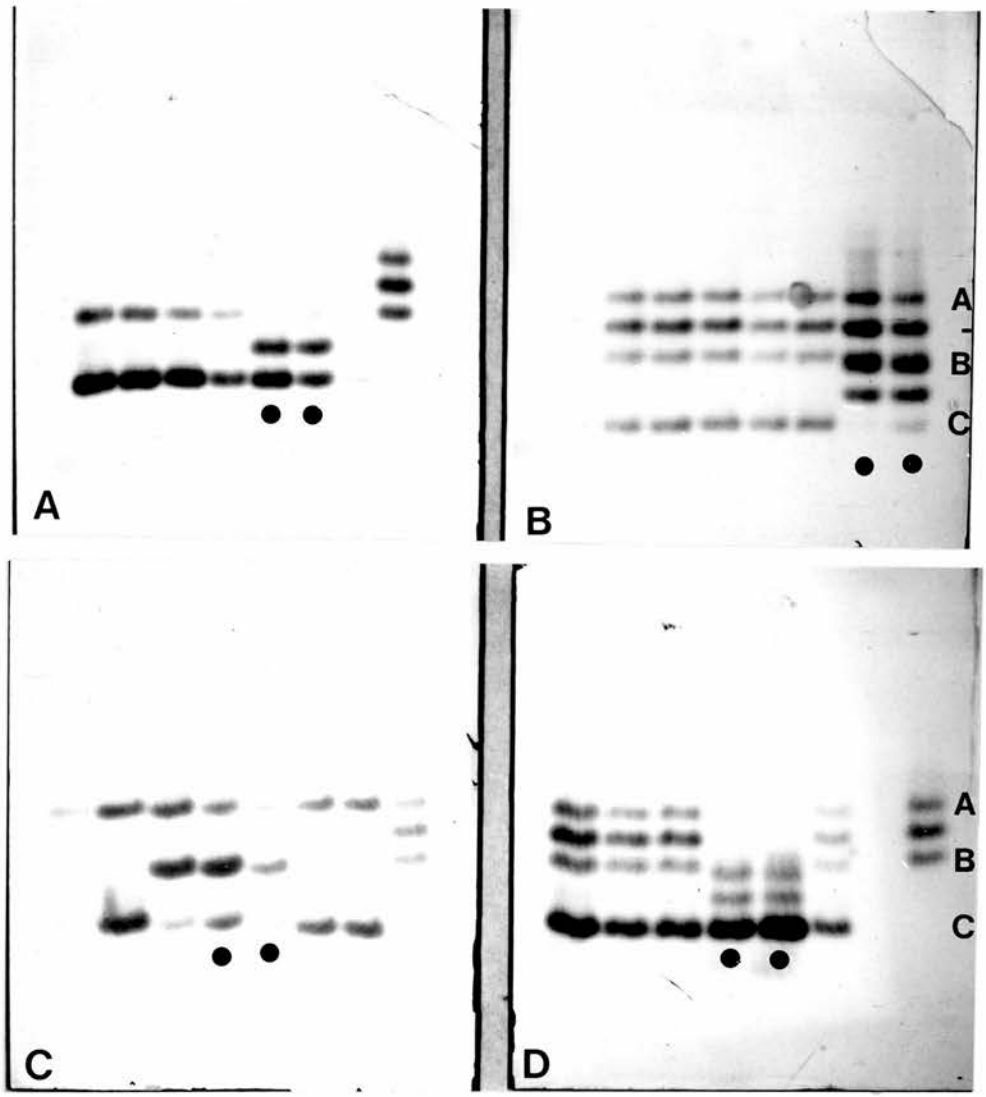
In D, only 3 bands are present compared to 5 in B (chimaera with same GPI phenotype)

Key on gel B & D

A = GPI-1A

B = GPI-1B

C = GPI-1C


A
B
C
A
B
C
A
B
C
D

GPI-1C band. The GPI-1A allozyme might be present but below the level of detection.

All the tissues assayed from the putative homozygous null chimaera (AK2) produced only GPI-1C providing further evidence that it was a homozygous null chimaera. A preliminary *in situ* hybridisation analysis was also performed on a selection of the tissues (Table 10.6). The morphology of some of the tissues was poor due to the female having died prior to removal of the tissues. Transgenic cells were not detected in all of the tissues analysed, this may have been due to the poor preservation of the tissue, or the fact that certain tissues within the chimaera were non-chimaeric, or it could be that there was only a low contribution of *m/m* cells to the tissues and because only a few sections were analysed these cells were not present. The homozygous null cells may be selected against in some tissues during development. The brain, thymus, uterus, bladder, anterior small intestine (region I) and liver left lateral lobe all had transgenic signal present but this was at a low level. Also in a sample of blood taken from this chimaera at 5 weeks of age approximately 8% of the cells (31/399) carried the transgenic marker, which roughly corresponded to the proportion of pigmented cells in the coat. The right ovary and oviduct were also sectioned for *in situ* hybridisation analysis to try to identify homozygous null oocytes (by the presence of the transgenic marker). Only one was observed (Fig 10.3). The transgenic marker was also observed in a proportion of the granulosa cells surrounding the oocytes, but not in the cells of the corpora lutea

10.4 DISCUSSION

Eleven offspring were produced from the three transfers. The GPI genotypes of these were 4 GPI-1C, 1 GPI-1A+C, 3 GPI-1B + C and 3 GPI-1AB + C. Of the 11 chimaeras that were born, two looked like candidates to be homozygous null chimaera (AK1 & 2). Test matings were set up to verify the suspected chimaeric genotypes. Chimaera AK1 produced only *c/c* albino offspring, therefore this mouse

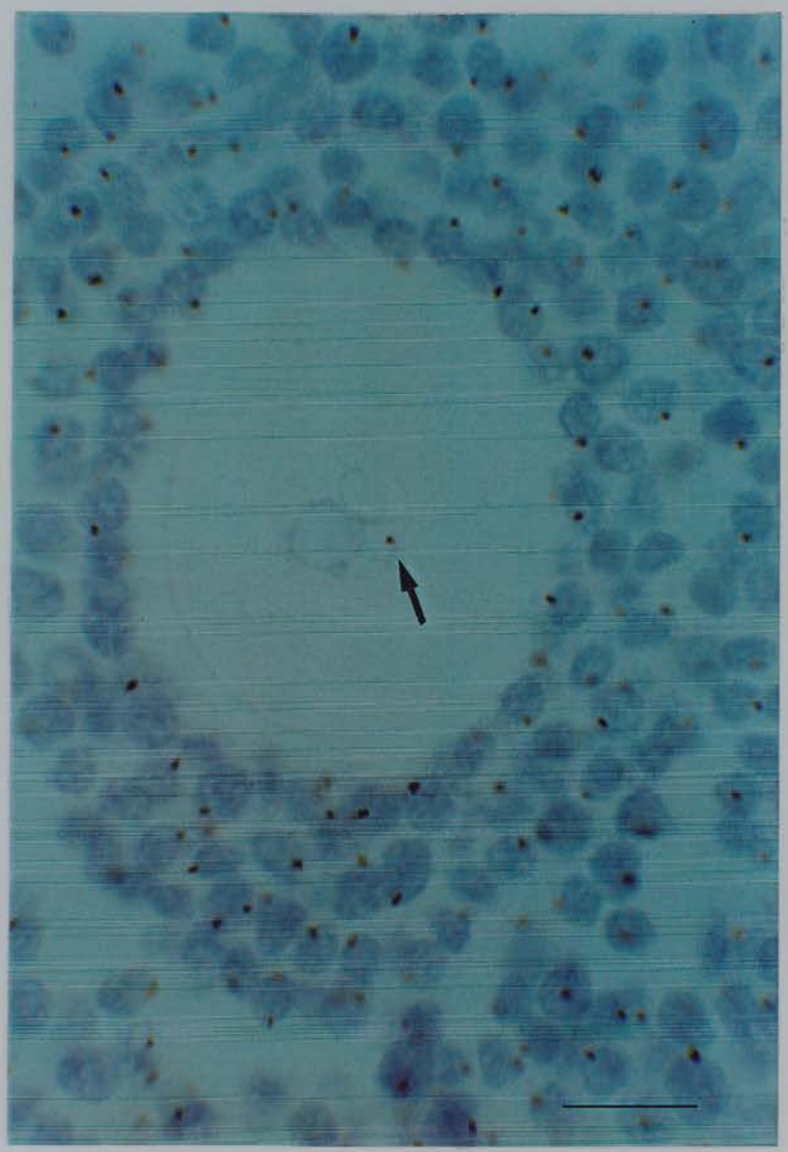


Fig 10.**3** Follicle from chimaera AK2, after *in situ* hybridisation to a β-globin transgene, note transgenic signal in the oocyte (arrow). Scale bar = 20μm.

is still classed as apossible homozygous null chimaera. The breeding experiments confirmed that AK2 was a homozygous GPI null chimaera.

This provides evidence that GPI homozygous null cells can survive to term and to adulthood in aggregation chimaeras. AK2, the *m/m* ↔ *c/c* chimaera was fertile and passed on the null allele to offspring. A limited *in situ* hybridisation analysis of the ovary identified one homozygous null oocyte. If the homozygous null oocytes cannot use glycolysis to obtain energy because they lack GPI then how do they survive? It has been demonstrated that oocytes (denuded of follicle cells) cannot utilize glucose but use pyruvate as the main energy source (Biggers *et al*, 1967). However, if follicle cells are present, then glucose can be metabolized (Donahue & Stern, 1968). This demonstrates that glycolysis is active in follicle cells but not oocytes and illustrates that follicles cells can communicate with the oocyte. Follicle cells also communicate with each other via gap junctions allowing the exchange of low molecular weight molecules (Anderson & Albertini, 1976, and Gilula et al 1978). More recently it has been shown that follicle cells rely predominantly on glycolysis for energy provision between mid preantral and preovulatory stages (Boland *et al*, 1994). Therefore follicle cells are able to supply the oocyte with pyruvate or other metabolites for energy production. If this pathway of metabolite transfer is available to the oocyte, it is perhaps not surprising that homozygous null (*m/m*) oocytes are able to survive. But this would depend on an adequate proportion of the follicle cells being wild type. The *in situ* hybridisation of the ovary did identify homozygous null granulosa cells (because of the presence of the transgenic marker). If all of the follicle cells surrounding the oocyte were homozygous for the null allele, the oocyte would be unable to survive as the follicle cells would be unable to utilize glycolysis and supply the oocyte with pyruvate. There may be a threshold level that homozygous null follicle cells cannot exceed or follicle development is impaired. Therefore there may be selection against homozygous null follicle cells in the ovary because they cannot utilize glucose to sustain their own development.

The majority of the homozygous null chimaeras identified in Chapters 5 and 6 contained 10% or less transgenic cells in the fetus (ie brain and tail) with the exception of Vx51 which had approximately 30%. At the time of aggregation it would be expected that the two 8-cell embryos have the ability to contribute equally to all the tissues of the resultant chimaera. Only a small number of chimaeras were allowed to develop to term so it is not clear whether there is an overall decline in the proportion of *m/m* cells between 12.5 days and adulthood. However, the one *m/m* ↔ *c/c* chimaera identified shows that *m/m* cells are not eliminated before adulthood. Results from eye pigment in fetal chimaeras suggests that there may be selection against homozygous null cells from the time of aggregation until 12.5 days (Chapter 8). If so, the selection would probably continue beyond 12.5 days. A study looking at the proportion of homozygous null cells contributing to various tissues at an earlier stage in development may determine whether selection against the *m/m* cells occurs in all tissues at an earlier stage of development.

This study identified one female adult chimaera as a possible homozygous null chimaera and one female as a definite homozygous null. This provides evidence that homozygous null cells can survive in *m/m* ↔ *c/c* chimaeras to term and beyond. The female was fertile and subsequent offspring were produced that were heterozygous for the null allele. It would be of interest to continue this study to try to produce male homozygous null chimaeras to investigate whether homozygous *m/m* spermatocytes were viable and produced functional spermatozoa.

In situ hybridisation analysis on a selection of tissues from the probable homozygous null chimaera (AK2) showed that not all of the tissues were chimaeric. It is not known whether the homozygous null cells failed to contribute to the formation of these tissues or whether they were selected against during development. This question could be addressed by comparing the various tissues at 12.5 days gestation (in the chimaeras described in Chapter 5) and at term although AK2 had roughly the

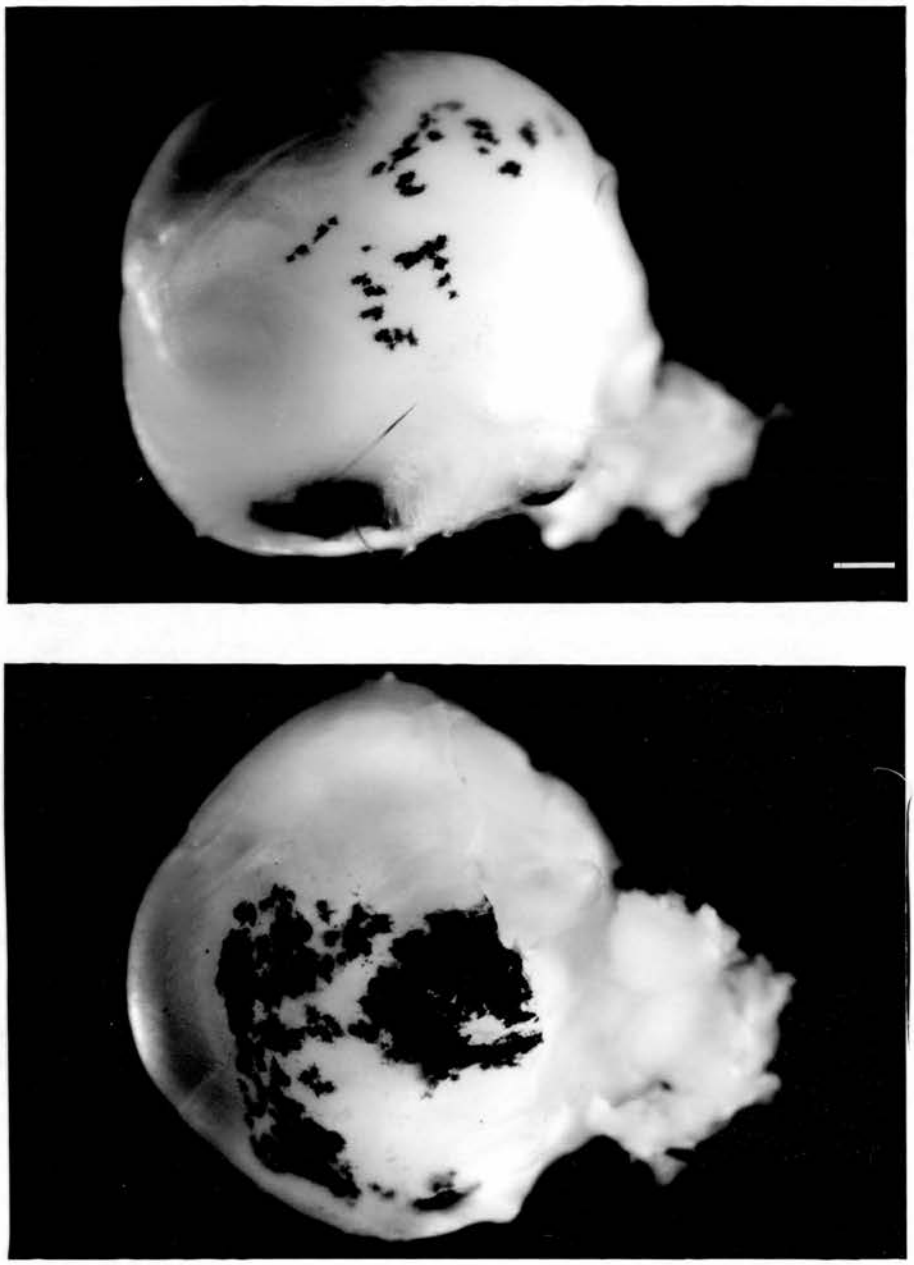


Fig 10.3 Two views of right eye of homozygous *m/m* ↔*c/c* chimaera, AK2.
Aproximately 15% pigment was estimated as being present.
Scale bar = 3mm

same contribution of *m/m* cells detected in the coat (as pigment) and in the blood (by presence of trangenic marker) as the 12.5d homozygous null chimaeras. Another study looking at earlier stages in development of homozygous null chimaeras may give a better indication of whether selection against *m/m* cells is occuring. A further *in situ* hybridisation experiment performed on tissues from AK1 may identify transgenic cells in this chimaera.

Preliminary results have shown that the presence of some homozygous null cells within tissues of ectodermal (brain), mesodermal (ovary and oviduct, see Table 10.7) and endodermal (small intestine and liver) origin. Their presence within these tissues does not appear to disturb the functioning of these organs. A more detailed study could be performed to determine the exact distribution of the homozygous null cells within various tissues.

By producing more female homozygous null chimaeras, a more thorough analysis of the ovary could be undertaken to determine whether there is any selection against homozygous null follicle cells. However, the single confirmed homozygous *m/m* $\leftrightarrow$ *c/c* female chimaera studied here has demonstrated for the first time that homozygous null female germ cells are both viable and functional in chimaeras.

CHAPTER 11

SUMMARY AND DISCUSSION

11.1 THE DEVELOPMENTAL POTENTIAL OF HOMOZYGOUS NULL EMBRYOS

One of the initial aims of this thesis was to identify the first sign of developmental abnormality apparent in homozygous GPI null embryos. A previous study by West, (1993) suggested that the homozygous null embryos failed to develop beyond gastrulation. The histological study undertaken (Chapter 2) identified an abnormal class of embryos that failed to develop beyond the egg cylinder stage. These abnormal embryos produced only a small amount of mesoderm at gastrulation, and expanded to form an empty sac like structure, composed of embryonic and extraembryonic tissues. The work presented here provides evidence that the mutant homozygous null embryos have undergone developmental failure rather than embryonic failure, because a fetus never developed within the empty sac (Fig 2.5).

Many other mutants that arrest during gastrulation have been described (see Chapter 1) and the genetic cause of some of these has also been elucidated. Although the reason for developmental failure in these mutant homozygous GPI null embryos is most likely due to metabolic failure, other genes may be affected. It would be interesting to investigate the expression of genes known to be required during gastrulation to see whether expression is absent or ectopic in the homozygous GPI null embryos. For example, *nodal* RNA is first expressed in the primitive streak at the time of mesoderm formation and becomes localized in the node at the anterior end of the primitive streak as gastrulation proceeds (Conlon *et al*, 1994). Looking for *nodal* mRNA or *goosecoid* (another primitive streak marker, Blum *et al* 1992) expression in the homozygous GPI null embryos may identify whether normal

primitive streak development occurs in these mutant embryos. Also looking at mesodermally expressed genes such as *Brachyury*, may give an indication of where the small amount of mesoderm that actually forms originates from.

An attempt was made to analyse the metabolic activity in the homozygous null embryos to try to identify whether different parts of these embryos (outside tissues compared to inside) metabolized energy in different ways. A previous study had shown that mitochondrial morphology changed when embryos were cultured in various O_2 concentrations (Morris & New, 1979). It was thought that by looking at the mitochondrial morphology in the different tissues of the mutant embryos might give an insight into whether different metabolic pathways were being used in the different tissues. As cytochrome-c oxidase is a mitochondrial marker, the activity of this enzyme was also investigated using a protein assay and mRNA *in situ* hybridisation. A very preliminary study on normal embryos did not provide any useful information and therefore the study was not extended to the homozygous GPI null embryos.

An immunological model of embryonic death was also examined. The CBA/J x DBA/2 system is used in studies as a model of recurring spontaneous abortion. A high resorption rate has been previously reported in these matings (Clark *et al*, 1980, Baines *et al*, 1994). Comparison of the development of the homozygous GPI null embryos and the CBA/J x DBA/2 resorbing fetuses showed that the CBA/J x DBA/2 resorbing fetuses were dying at a later stage, at approximately 9.5 d.p.c. (Chapter 4). A fetus formed, but then degenerated and died probably due to being attacked by an infiltrate of maternal lymphocytes.

The resorbing fetuses observed from the three types of CBA/J matings in this study (CBA/J x DBA/2, CBA/J x BALB/c and CBA/J x CBA/J) were all similar in appearance which could mean that although the cause of the resorption of these embryos is likely to be different the resorption process follows a similar pattern. The

incidence of resorptions in CBA/J x DBA/2 matings was not consistently higher than control CBA/J x BALB/c matings (which undermined the basis for this study).

An *in situ* hybridisation analysis was attempted to look at the expression of TNF-α mRNA in both GPI homozygous null embryos and resorbing embryos from the CBA/J x DBA/2 model. The *in situ* technique had not been optimised and therefore the results obtained were very preliminary and no conclusions about whether the expression of TNF- α was similar and therefore was a consequence of the resorption process rather than the cause of the death observed in the CBA/J x DBA/2 matings.

11.2 THE DEVELOPMENTAL POTENTIAL OF HOMOZYGOUS NULL CELLS

Once the homozygous GPI null embryos had been characterized, the next step was to produce aggregation chimaeras between normal embryos and homozygous null embryos to determine whether homozygous null cells could survive beyond the time of fetal death observed in the homozygous null embryos. Studying the fate of mutant cells in chimaeric embryos produced by the aggregation of mutant and normal embryos can give information on whether the mutant is cell autonomous or whether it can be rescued by contact with normal wild type cells. In previous chimaera studies, it was found that cells homozygous for the lethal yellow mutation (A^y) could not be rescued in chimaeras (Papaioannou & Gardner, 1976, Barsh *et al*, 1990), but cells homozygous for the 413-d insertional mutation were rescued in injection chimaeras (Conlon *et al*,1991), as were some trisomies (Ts 12, Fundele *et al*, 1985, Ts 15, Epstein *et al*, 1984, Ts 16, Cox *et al*, 1984 and Ts 17, Epstein *et al*, 1982) and parthenogenetic cells (Fundele *et al*, 1990).

Because of the difficulty in identifying homozygous null embryos at the 8-cell stage, all of the embryos produced from crossing the two heterozygotes were aggregated to

normal embryos. A quarter of these embryos should be homozygous for the null allele of *Gpi-1s*. Homozygous GPI null chimaeras were identified at 12.5 d.p.c.

a) Fetal heterozygous null chimaeras

Heterozygous GPI null (*a/m* ↔*c/c* and *b/m* ↔*c/c*) chimaeras were also identified. Results from these chimaeras suggested that heterozygous null (*+/m*) cells are at a selective disadvantage in the pigmented retinal epithelium within these chimaeras compared with normal cells in homozygous normal chimaeras.

b) Fetal homozygous null chimaeras

In the homozygous GPI null chimaeras, *m/m* cells also appeared to be selected against in certain tissues. Pigment analysis in the eye demonstrated that homozygous null chimaeras had less pigmented cells present compared with homozygous normal chimaeras but not heterozygous null chimaeras. The spatial distribution of pigmented homozygous *m/m* cells, heterozygous *+/m* cells and homozygous normal +/+ cells were compared (Chapter 9). Although there is a difference in the contribution of homozygous null (*m/m*) and heterozygous null (*+/m*) cells that can contribute to the pigmented retinal epithelium compared with homozygous normal (+/+) cells, there is no significant difference in the mean corrected patch size these three types of cells can form. The pigmented retinal epithelium is a single layer of cells, the homozygous null cells may be able to acquire nutrients from the mesenchyme below and the neural retina above, and therefore survive in patches equivalent in size to the patches formed in heterozygous null and homozygous normal chimaeric eyes.

In situ hybridisation of probable (and probable plus possible) homozygous null chimaeras suggested the possibility that *m/m* cells were being selected against within tissues of the primitive ectoderm lineage. Selection was not apparent between the fetus and the membranes but between tissues of the primitive ectoderm lineage and the primitive endoderm. Selection against abnormal cells within other types of

chimaeras (Nagy *et al* 1987, Fundele *et al*, 1990) and selection against HPRT$^-$ cells in X-inactivation mosaics (Ansell *et al*, 1991) have been demonstrated previously.

A more thorough *in situ* analysis of the homozygous normal (+/+↔ *c/c*) chimaeras and all of the heterozygous null (+/*m* ↔ *c/c*) chimaeras could be made. As time was limited, only a small selection of heterozygous null chimaeras that had comparable eye pigmentation to the homozygous null chimaeras were analysed. However, if all the chimaeras were analysed by the transgenic marker, the proportion of the transgenic cells identified in different tissues could be compared between the three groups. This may identify whether the heterozygous null (*a/m* or *b/m*) cells are selected against in certain tissues or as may be the case, lineages compared to normal cells (*a/b*) in homozygous normal chimaeras. Both heterozygous null and homozygous normal chimaeras can be compared to the homozygous null chimaeras.

c) Adult chimaeras

A small series of chimaeras were allowed to develop until term. A single female chimaera was identified as a homozygous GPI null chimaera and passed on the null allele to subsequent offspring. This female was approximately 10% pigmented and had approximately 8% transgenic cells detected in a blood sample. This is comparable to the homozygous null chimaeras identified at 12.5 days and suggests that very little, if any selection against homozygous *m/m* cells occurs between 12.5 days and term and beyond. Selection against *m/m* cells may therefore be occuring at earlier stages of development. In an attempt to try to address this idea, a further chimaera experiment based on series (ii), analysing homozygous null chimaeras at an earlier stage of development may identify when selection against homozygous null cells occurs.

If more adult chimaeras were produced it may be possible to identify male homozygous GPI null chimaeras, and an investigation of whether homozygous *m/m*

spermatocytes were viable and produced functional spermatozoa could be undertaken.

The one female homozygous null chimaera identified in this study produced viable and functional germ cells. The production of more female homozygous GPI null chimaeras would enable the investigation of whether selection against homozygous null follicle cells occurs within the ovary.

CONCLUSIONS

The results presented in this thesis have demonstrated that homozygous GPI null embryos do not develop beyond gastrulation. However, homozygous null cells can be rescued and survive until 12.5 days of gestation in aggregation chimaeras in various tissues and also until term and beyond. One female homozygous null chimaera was identified and developed to adulthood. Offspring from this female were heterozygous for the null allele of *Gpi-1s* demonstrating that homozygous female germ cells are both viable and functional in aggregation chimaeras.

APPENDICES

7

COMPARISON OF GPI AND IN SITU HYBRIDISATION ANALYSES

A comparison was made of the GPI and *in situ* hybridisation estimates of (GN x NUL) cells in the 2 *a/m* ↔*c/c* and 4 *b/m* ↔*c/c* chimaeras that were analysed (Table A7.1). There are some cases where GPI-1A (or GPI-1B) was detected in the tissues but no transgene was seen, and cases where no GPI-1A (or GPI-1B) was detected but transgene was found. No positive correlation was observed between the GPI and transgene data, but the sample size was small.

When the corrected % GPI-1A (or GPI-1B) and transgene were compared using the Wilcoxon signed ranks test, there was no significant difference in the means of either the tail or placenta (Table A7.1). The yolk sac was sampled as a whole for the GPI electrophoresis analysis but for the *in situ* analysis, the yolk sac mesoderm and endoderm were scored separately. The corrected % GPI-1A (or GPI-1B) was compared with the transgenic data of both the yolk sac mesoderm and yolk sac endoderm and also the mean of these two. The corrected % GPI-1A (or GPI-1B) related most closely with the % Tg detected in the yolk sac endoderm. The corrected % GPI-1A (or GPI-1B) for the yolk sac was significantly different from the mean % Tg detected in the yolk sac mesoderm and yolk sac endoderm but not the yolk sac mesoderm or endoderm (Table A7.1).

Inconsistencies in the detection of transgene and the % GPI-1A (or GPI-1B) illustrates the problem of sampling only small pieces of tissue. A low proportion of cells may not have been detected in one of the analyses but detected in the other (sampling effects or perhaps because of the GPI assay detection limit of 3%). Cells of like genotype could grow in large patches or clusters of smaller patches. A mixed population of cells observed in one sample and not in the other could be due to a single patch being sampled for one analysis and both types of cells being sampled for

the other. This patch-like growth of cells has been demonstrated in the yolk sac endoderm of chimaeras produced using differences in malic enzyme activity between normal *Mod-1*$^{+}$ / *Mod-1*$^{+}$ and mutant *Mod-1*^{n} /*Mod-1*^{n} cells (Gardner, 1984). Wild type cells in the yolk sac endoderm were found in discrete patches whereas in the yolk sac mesoderm a more diffuse dstribution of normal cells was observed. Clusters of smaller patches have been demonstrated in the retinal pigmented epithelium when the proportion of pigmented cells was low (Sanyal & Zeilmaker, 1977). Inconsistencies in the results (Table A7.1) from the placenta could be due to the maternal part (the same GPI genotype as the (CF1 x CF1) component of the chimaera) being sampled for the GPI analysis and fetal cells being sampled for the *in situ* analysis, because the maternal part of the chimaera would be obvious because of the different histology.

Table A7.1 Comparison of GPI and *in situ* hybridisation estimates of % (GN x NUL) cells.*

Chimaera No.	Tail		Yolk Sac				Placenta	
	Corrected % GPI-1 (A or B)	Corrected % Tg	Corrected % GPI-1 (A or B)	Corrected % Tg			Corrected % GPI-1 (A or B)	Corrected % Tg
				Yolk sac mes	Yolk sac end	Yolk sac mean		
Vx24	0.00	0.00	23.88	0.00	0.41	0.21	6.73	0.00
Vx46	7.46	8.22	22.61	0.46	18.52	32.26	0.00	7.26
Vx27	12.78	26.47	18.84	9.06	42.71	25.88	44.24	21.02
Vx49	16.31	13.27	16.71	3.56	0.39	1.98	0.00	12.28
Vx19	20.93	0.00	27.27	0.00	17.96	8.98	18.44	0.00
Vx40	24.01	0.44	79.82	2.84	11.57	7.21	70.33	65.74
Mean±S.E	**13.58±3.62**	**8.07±4.30**	**31.52±9.78**	**2.65±1.42**	**15.26±6.39**	**8.96±3.72**	**23.29±11.60**	**17.72±10.14**
S.D.	8.86	10.53	23.95	3.49	15.66	9.11	28.41	24.83
Spearman Rank Correlation r_S	-0.014 P=0.975		-0.557 P=0.213	0.029 P=0.949	-0.143 P=0.749		0.514 P=0.250	
Wilcoxon signed rank	P=0.345		P= ***0.028***	P=0.249	P=***0.046***		P=0.463	

* GPI estimate of % (GN x NUL) cells is corrected for greater lability of GPI-1C enzyme and expected lower activity of GPI-1A or GPI-1B in *a/m* and *b/m* cells compared *a/a* and *b/b* cells and also for maternal contamination of placenta. In situ hybridisation estimate (% positive cells) is corrected according to % positive cells in hemizygous Tg/+ fetal heads.

(a) CONTROL +/*m* and +/+ CHIMAERAS

The relationships between the % eye pigment and the % transgene detected were tested using Spearman Rank correlations (Table A8.1) to determine whether correlations were observed within or between the lineages analysed. Correlations between tissues within the primitive ectoderm lineage have been demonstrated previously (West *et al*, 1984, James *et al*, 1993, and West & Flockhart, 1994, West *et al*, 1995). In this group of chimaeras, a significantly positive correlation was observed between the % eye pigment and the proportion of transgenic cells contributing to the tail. This correlation was also observed in the heterozygous null chimaeras in the previous Chapter. The tail and the amnion, tail and yolk sac mesoderm, and amnion and the yolk sac mesoderm were also significantly positively correlated (Table A8.1, Appendix). The only significantly positive correlation observed between the lineages was between the brain and the yolk sac endoderm (Table A8.1, Appendix).

In order to determine whether the contribution of transgenic cells to the different tissues within the primitive ectoderm lineage and between the tissues of the primitive ectoderm and primitive endoderm and trophoblast lineages of the conceptuses are significantly different Wilcoxon signed rank tests were applied (Table A8.2 and histograms plotted Fig 8.2A. If the contribution of transgenic cells to different tisues was significantly different, it could suggest that tissue-specific selection was occurring. However, because in this group, both heterozygous null (+/*m* ↔ *c*/*c*) and homozygous normal (+/+↔ *c*/*c*) chimaeras are pooled, any effect of +/*m* cells may not be statistically significant because of the presence of +/+ transgenic cells in the homozygous normal chimaeras. The contribution of transgenic cells to the tail and amnion were significantly greater than the yolk sac mesoderm (Table A8.2). This

low proportion of transgenic cells detected in the yolk sac mesoderm is apparent from the histogram (Fig. 8.2A).

When comparing the proportion of transgenic cells detected between the lineages, significant differences were observed in the contribution of transgenic cells to the amnion and yolk sac mesoderm compared to the yolk sac endoderm and placenta (Table A8.2).

Table A8.1 Spearman Rank correlation coefficients (r) with Probability (P) values below for % Tg detected in different tissues in *+/m ↔c/c* or *+/+ ↔c/c*, possible and probable *mm/ ↔c/c* and probable *mm/ ↔c/c* chimaeras from series (i) & (ii)

		+/m ↔c/c *+/+ ↔c/c* N=12	Prob *mm/ ↔c/c* N=14	Prob and poss *m/m ↔c/c* N=28
Within primitive ectoderm lineage				
Pigment versus Brain		0.427 P=0.157	0.734 P=***0.008***	0.692 P<***0.0001***
	Tail	0.608 P=***0.044***	0.678 P<***0.001***	0.775 P=***0.007***
	Amnion	0.547 P=0.070	0.749 P=***0.007***	0.645 P=***0.001***
	YS mes	0.462 P=0.126	0.645 P=***0.020***	0.541 P=***0.005***
Brain versus	Tail	0.462 P=0.126	0.615 P=***0.033***	0.745 P<***0.001***
	Amnion	0.544 P=0.071	0.624 P=***0.024***	0.767 P<***0.001***
	YS mes	0.495 P=0.101	0.805 P=***0.004***	0.687 P<***0.001***
Tail versus	Amnion	0.722 P=***0.017***	0.835 P=***0.004***	0.820 P<***0.0001***
	YS mes	0.713 P=***0.018***	0.745 P=***0.010***	0.705 P<***0.001***
Amnion versus YS mes		0.953 P=***0.002***	0.551 P=***0.047***	0.705 P<***0.001***

Table A8.1 continued

	+/m ↔c/c *+/+ ↔c/c* N=12	Prob *mm/ ↔c/c* N=14	Prob and poss *m/m ↔c/c* N=28
Between lineages			
YS end versus Pigment	0.287 P=0.342	-0.226 P=0.414	0.194 P=0.313
Brain	0.622 P=***0.039***	0.176 P=0.526	0.401* P=***0.041***
Tail	0.483 P=0.110	-0.148 P=0.607	0.186 P=0.343
Amnion	0.533 P=0.077	-0.134 P=0.629	0.194 P=0.324
YS mes	0.490 P=0.105	0.142 P=0.609	0.457* P=***0.018***
Placenta versus Pigment	0.350 P=0.246	0.545 P=***0.049***	0.453* P=***0.019***
Brain	0.105 P=0.728	0.431 P=0.120	0.598 P=***0.0023***
Tail	0.531 P=0.078	0.556 P=0.054	0.551 P=***0.0049***
Amnion	0.544 P=0.071	0.576 P=***0.038***	0.573 P=***0.0035***
YS mes	0.427 P=0.157	0.173 P=0.534	0.365 P=0.058
YS end	0.147 P=0.626	-0.264 P=0.342	0.278 P=0.149

Probabilty values were taken to be statisically significant when $P<0.05$
* r_S value <0.5 but $P<0.05$

(b) PROBABLE HOMOZYGOUS NULL CHIMAERAS

The proportion of *m/m* cells detected correlated very significantly positively within the primitive ectoderm lineage (Table A8.1). Significantly positive correlations were also observed between the placenta and eye pigment and also the placenta and amnion but these were weaker than the within lineage correlations (Table A8.1). In order to determine whether there was any significant difference in the proportion of pigmented cells in the eye and transgenic cells (i.e. homozygous null cells) detected in the tissues analysed by *in situ* hybridisation, pairwise Wilcoxon signed rank tests were applied (Table A8.2). This would give an indication of whether tissue-specific selection against *m/m* cells was occuring. Within the primitive ectoderm lineage, there were no significant differences in the contribution of homozygous null cells to the different tissues (detected as pigment in the eye and by the transgenic marker elsewhere in the conceptus) analysed (Table A8.2). Comparisons between lineages revealed that the proportion of *m/m* cells contributing to the yolk sac endoderm was significantly greater than in the brain, amnion and yolk sac mesoderm and the placenta was significantly greater than in the yolk sac mesoderm (Table A8.2). The fairly consistent observation of higher contribution of *m/m* cells to the yolk sac endoderm compared to tissues within the primitive ectoderm lineage, suggests that there is selection against *m/m* cells in the primitive ectoderm lineage.

(c) PROBABLE AND POSSIBLE HOMOZYGOUS NULL CHIMAERAS

When the probable and possible homozygous null chimaeras are grouped the correlations observed between all the tissues within the primitive ectoderm lineage became more significantly positive (Table A8.1). Between the lineages, significantly positive correlations were observed between the yolk sac endoderm and the brain and the yolk sac endoderm and the yolk sac mesoderm, however the r_S values were <0.5. The placenta correlated significantly positively with pigment, brain, tail and amnion. In the case of pigment versus placenta although a significantly positive correlation was observed the r_S value was <0.5.

To test whether the difference in proportion of homozygous null cells contributing to tissues within the primitive ectoderm lineages or between the lineages was statistically significant, Wilcoxon signed rank tests were applied. Within the primitive ectoderm lineage, significant differences were observed between the pigment and yolk sac mesoderm and amnion and yolk sac mesoderm (Table A8.2). In the three groups of chimaeras analysed, the yolk sac mesoderm had the lowest contribution of transgenic cells. The proportion of *m/m* cells contributing to the yolk sac endoderm was significantly greater than in the brain, tail, amnion and yolk sac mesoderm, again suggesting lineage specific selection against *m/m* cells in the primitve ectoderm. The placenta was also significantly different from the yolk sac mesoderm.

Table A8.2 Probability (P) values from Wilcoxon sign rank tests for corrected Tg data from series (i) & (ii) for *+/m ↔c/c* or *+/+ ↔c/c*, probable *m/m ↔c/c* , and possible plus probable *m/m↔c/c* .

		+/m ↔c/c *+/+ ↔c/c* N=12	Prob *mm/ ↔c/c* N=14	Poss and prob *m/m ↔c/c* N=28
Within primitive ectoderm lineage				
Pigment versus	Brain	0.859	0.286	0.376
	Tail	0.076	0.203	0.196
	Amnion	0.799	0.333	0.648
	YS mes	0.594	0.059	***0.028***
Brain versus	Tail	0.139	0.678	0.842
	Amnion	0.799	0.799	0.653
	YS mes	0.161	0.139	0.055
Tail versus	Amnion	0.110	0.859	0.256
	YS mes	***0.028***	0.173	0.096
Amnion versus	YS mes	***0.005***	0.114	***0.034***
Between lineages				
YS end versus	Pigment	0.091	0.064	0.068
	Brain	0.091	***0.033***	***0.039***
	Tail	0.328	0.060	***0.034***
	Amnion	***0.023***	***0.013***	***0.013***
	YS mes	***0.012***	***0.003***	***<0.001***
Placenta versus	Pigment	0.059	0.790	0.520
	Brain	0.075	0.100	0.122
	Tail	0.575	0.075	0.055
	Amnion	***0.016***	0.110	0.126
	YS mes	***0.013***	***0.041***	***0.011***
	YS end	0.480	0.470	0.627

Probabilty values were taken to be statisically significant when P<0.05

9

CORRECTION OF OBSERVED MEAN PATCH LENGTH.

In a random one dimensional sequence of black and white clones, the number of patches is equal to the number of clones forming the left hand end of a patch. A clone will be the left hand end of a black patch if it is black and its left hand neighbour is white. For any clone this has the probability p(1-p) where p is the proportion of black clones.

Thus, in a line of N clones the expected number of patches is Np(1-p).

$$\text{Expected mean number of clones per patch} = \frac{\text{Expected number of black clones}}{\text{Expected number of black patches}}$$

$$= \frac{Np}{Np(1-p)}$$

$$= \frac{1}{(1-p)}$$

$$\text{Corrected mean patch length} = \text{Estimated clone length}$$

$$= \frac{\text{Observed mean patch length}}{\text{Expected mean number of clones per patch}}$$

$$= \frac{\text{Observed mean patch length}}{1/(1-p)}$$

I. MOUSE STRAINS

Abbreviated stock name	Details	Genotype	
		Gpi-1s	β-globin transgene
BALB/c	BALB/c/Eumm	*a/a*	-/-
BF_1	(C57BL female x CBA male)F1 hybrid	*b/b*	-/-
BGO	C57BL/Ws-*Gpi-1s*^{a}/*Gpi-1s*^{a}-*m1H*	*a/m*	-/-
or	C57BL/Ws-*Gpi-1s*^{b}/*Gpi-1s*^{a}-*m1H*	*b/m*	-/-
C57BL	C57BL/Ws	*b/b*	-/-
CALB	BALB/c-*Gpi-1s*^{c}/*Ws*	*c/c*	-/-
CBA	CBA/Ca	*b/b*	-/-
CBA/J	CBA/J/Ola/Hsd		-/-
*CC	C57BL-*Gpi-1s*^{c}, *c/Ws*	*c/c*	-/-
*CF_1	(CC female x CALB male)F1 hybrid	*c/c*	-/-
DBA/2	DBA/2/Ola		-/-
GN	BF1 x BGO (*Gpi-1s*^{a})	*b/o*	-/-
NUL	(BGO x TGA, or BGO x TGB)	*a/m*	+/+
		b/m	+/+
TGA	CLO (strain 83) x BF1	*a/a*	+/+
TGB	CLO (strain 83) x BF1	*b/b*	+/+

* See West *et al*, 1995.

II. ELECTRON MICROSCOPY

PROCESSING

DDSA	Dodecenyl Succinic Anhydride
DMP30	2,4,6 - tris (Dimethylaminomethyl) phenol

1. Fixation	Fix in 3% gluteraldehyde (pH 7.2) in 1% Phosphate buffer 1-24hr.
2. Wash	Wash in 0.5% sucrose in 0.1% phosphate buffer for 30 mins.
3. Postosmication	1% osmium tetroxide in PBS for 1-2hrs.
4. Washing bubbling	Wash in 10% alcohol for 2 x 15 min Prevents in the 100% alcohol and removes the osmium.
5. Dehydration	100% alcohol, 3 x 30 min
6. 'Clearing'	Propylene oxide.(BDH) for 30 min. This is very volatile.
7. Embedding mixture 964))	Sol B 50: 50, araldite resin : DDSA (hardener HY Sol A 1: 2 stock solution, DMP30 (accelerator DY 064):dibutyl pthalate. 0.5 mls Sol A in 19mls of Sol B. Mix thoroughly. Place samples in this overnight.

8. Remove samples to fresh Sol B and heat in a 60^{o}C for 48hrs to polymerise.

9. Blocks sawn from the araldite are mounted on dowel rods with sealing wax.

10.Thin sections are cut on a Reichert Ultracut E microtome.

11.Staining	saturated solution of uranyl acetate in 59 % ethanol 10 - 15 min

Emmerse section in stain.

Rinse in 50% ethanol

0.2% lead citrate Float sections

Rinse in distilled H_2O

Mount sections on New 200 copper grids without a supporting membrane

III. IN SITU METHODS

a) CYTOCHROME OXIDASE IN SITU HYBRIDISATION

REAGENTS

Dig RNA labelling mix	Boehringer (1277 073)
Dig-11 UTP	Boehringer (1209 256)
Recombinant RNAsin Ribonuclease Inhibitor	Sigma (N2511)
T3 RNA polymerase	Sigma (P2083)
T7 RNA polymerase	Sigma (P2075)

5X Tsc buffer

DTT

To synthesize sense and antisense riboprobes, Bluescript plasmid (Stratagene) containing cDNA E1 (606bp) was purified and linearized using *Bam* HI (antisense) and *Hind* III (sense). RNA was synthesized with T7 or T3 RNA polymerase respectively. Probes were labelled with digoxigenin-UTP (Boehringer-Mannheim) as described previously (Millar *et al*, 1993).

Labelling the probe

Add to the probe

Dig RNA labelling mix	10ml
5X Tsc buffer	20ml
RNAsin	2ml
T3 or T7	2ml
H_2O	51ml
DTT	10ml

1.Incubate at 37^{o}C for 1hr.

2.Run gel to check labelling has occured

3. Precipitate with 12.5ml 4M $LiCl_2$ and 375ml, 95% EtOH overnight at -20^{o}C.

4. Spin down at 4^{o}C for 30 min.

5.Wash in 80% EtOH.

6. Spin for 5 min and take off supernatant.

7. Air dry and resuspend in 50ml H_2O and 2ml RNAsin.

REAGENTS

Fixative Bouins Fluid	500mls	40% formaldehyde
	100mls	acetic acid
	2000mls	picric acid

Fix samples in Bouins fluid for approximately 5hrs.

PK buffer	100mls	1M Tris (pH 8.0)
	100mls	0.5M EDTA (pH 8.0)
	Make up to 1litre with H_20.	
TEA	Add 14.9g Triethalomine to 800 mls H_20 pH to 8.0	
	Make up to 1litre with H_20.	
RNAse buffer	20mls	Tris (pH 8.0)
	200mls	5M NaCl
	4mls	0.5M EDTA
	Make up to 2 litres with H_20.	
SSC	0.15M NaCl	
	15mM Na CO_3[pH7]	
20x SSC	175.3g	NaCl
	88.2g	Sodium citrate
	800mls	H_20
	Dissolve the salts in the H_20, pH to 7.0	
	Make up to 1litre with H_20.	

20x STE	3M NaCl	17.5g
	0.05M Tris	10mls 0.5M Tris (pH 7.4)
	0.005M EDTA	1ml 0.5M EDTA
	Add to 100mls H_2O.	

50x Denhardts solution (stored in freezer) — 0.5g BSA (Sigma), 0.5g PVP (Polyvinylpyrrolidine, Sigma) 0.5g Ficoll (Pharmacia) in 50 ml H_2O.

Prehybridisation Buffer		Final concentration
	Deionised formamide	50%
	20x STE	4x
	50x Denhardts solution	1x
	Salmon sperm DNA(10mg/ml)	125µg/ml
	Yeast (10mg/ml)	125µg/ml
	H_2O	

Hybridisation Buffer — as above plus the following

10% Dextran Sulphate

50-200ng/ml digoxigenin (DIG)-labeled cRNA.

Normal sheep serum

X- Phos — 5-Bromo 4chloro 2-indolyl phosphate (Boehringer)

Levamisole — Sigma

<u>METHOD</u>

1. Dewax slides in xylene and rehydrate.

2. 0.2N HCl for 20 mins at R.T.

3. 2x 5min washes in DEPC treated H_2O.

4. 20 min in P.K. buffer containing 20mg/ml^{-1} P.K.at 37^{o}C.

5. 0.2% glycine at 4^{o}C for 10-30mins

6. Wash in TEA for 5 mins.

7. Acetylate in TEA for 10 mins at RT.

8. Remove excess moisture from slide using tissue paper. Do not let sections dry out.

PREHYBRIDISATION

9. Prehybridise for 2hrs at 50^{o}C (50 prehybrid Dig) Drop on to slide, no need for coverslips.

10. Hybridize at 50^{o}C overnight.. 10µl probe in 500µl hybridization buffer.

WASHES

1. 2 changes of 4 x SSC over 15 mins at R.T.

2. RNAse buffer with RNAse at 20µg/ml at 37^{o}C for 30mins.

3. 2x SSC at R.T. for 5mins.

4. 2x SSC at R.T. for 5mins.

5.30% formamide in 0.1x SSC at 40^{o}C for 15 mins. (1.75mls 20x SSC+ 105mls formamide in 350mls H_2O)

6. Wash in TBS for 5mins.

7. Block with normal sheep serum 1:4 parts TBS for 30 mins. Drain.

8. Anti-dig antibody 1:300 made up in 7 (6.6µl in 2ml).

9. 2x 10 min washes in TBS.

10. 1x 3min TBS pH 9.5/MgCl 500 mM MgCl + 8 H_2O

[1M Tris 1M NaCl

10ml Buffer

45µl NBT

35µl X-Phos

10µl Levanisole blocks endogenous alkaline phophatase activity.

Leave overnight.

Look at every few hrs until the desired colour of staining is achieved.

Wash in H_2O.

Abs. Alc. 20 secs

Xylene 20 secs.

Mount in eukit.

b) TNF α IN SITU HYBRIDISATION

Fixation	PLP	
	Component A	0.1M Lysine in 0.05M PBS, pH 7.4
	Component B	8% paraformaldehydein distilled H_2O
	Component C	Sodium Iodate ($NaIO_4$ Sigma S-4007)

To make Component A

1. Dissolve 1.827g Lysine Monohydrochloride (Sigma L-5626) in 50ml H_2O and adjust pH to 7.4 with 0.1M Na_2HPO_4.

2. Add an equal volume of 0.1M PBS (pH7.4) to give a 100ml final volume

3. Store at 4°C for up to a month.

To make Component B

1. Add 8g paraformaldehyde to 100ml H_2O and stir at approximately 60°C until solution is reasonably clear. Make solution clear by adding 1 to 3 drops of 1M NaOH.

2. Filter through a 0.45 micron filter.

3. Store at 4°C for up to a month.

Make up PLP fixation buffer as below (just prior to use).

Mix A and B in a 3:1 ratio and add C (21.4mg/10ml)

4% Paraformaldehyde	Add 4g of paraformaldehye to 100mls sterile PBS. Stir continuously on heating block (at 60°C until dissolved).

Make up fresh on morning of use.

1Kb DNA ladder

This is labelled as the negative control for TNF-α in situ hybridisation experiment.The bands of the ladder contain from 1 to 12 repeats of a 1,081-bp DNA

fragment. In addition to these 12 bands, the ladder contains vector DNA fragments that range from 75 to 1.636bp.

REAGENTS

Hybridization solution	30ml/slide	Final Concentration
	RNA	5 x10^5cpm
	20x SSC	10%
	Formamide	50%
	50x Denhardts	1%
	100mM DTT	10mM
	Salmon sperm DNA (10mg/ml)	1mg/ml
	E. coli tRNA (10mg/ml)	0.1mg/ml
	H_2O	
Harris' Haematoxylin	BDH	
Acid/alcohol	70% alcohol: Conc. HCl , 99:1	
Scotts Tap water	2g Potassium bicarbonate, 20g Magnesium sulphate, 1l H_2O.	
Eosin solution, 3:1. Add	1%aqueous eosin (BDH) solution: 1%alcoholeosin 0.05% acetic acid before use.	

METHOD

1.De wax slides in 2 washes of xylene for 5 min each.

2 Rehydrate through a series of alcohols (100%, 95%, 70%, 5 min each).

3. Wash in 2 x SSC for 10 min

4. Immerse in 0.005% protease for 2.5 min at 37°C

5. Post fix in 4% paraformaldehyde for 10 min at 4°C.

6. Acetylate the slides in 0.25% acetic anhydride, 0.1M triethanolamine buffer [pH8.0] for 10 min at room temperature.

7.Immerse the slides in 0.1 M glycine, in 0.1M Tris-HCl [pH7.4] for 10 min.

8. Wash briefly in 2x SSC.

9.Dehydrate the slides in 70%, 95% and 100% ethanol and air dry for approximately 1hr.

HYBRIDISATION

10. A prehybridization step is not required. Add 30µl of the hybridization solution per slide, coverslip and seal with rubber cement. Hybridisation was carried out at 48^{o}C overnight.

WASHES

11. Wash slides in 1 x SSC/50 % formamide at 42^{o}C for 30 min.

12. 0.1 x SSC for 10 min at room temperature.

AUTORADIOGRAPHY

This is carried out in a dark room.

13. Slides are dipped into a liquid photographic emulsion, nuclear track K5 (Ilford Limited, Mobberley, Cheshire, UK), diluted with an equal volume of distilled water (40-42^{o}C).

14. Air dry upright overnight

15. Transfer to a light sealed box and expose for 10 - 14 days at 4^{o}C.

16. Develop the slides as follows	Kodak D-19 developer	1.5min
	Distilled water	15sec
	Kodak fixer	5min
	Distilled water	2 x 5min

17. Stain slides (as before)

18. Mount slides with eukit

c) β GLOBIN IN SITU HYBRIDISATION

Digoxygenin Labelling of Probes

The β globin probe is derived from the plasmid (pMBJ) which was inserted into the strain 83 transgenic mice (Lo, 1986). The probe contains 1000 copies of the mouse β globin gene. The plasmid pMßð2 was linearised by incubation at 37°C with *Eco*RI (10 units/µl) for 90min. Phenol/chloroform extraction was performed and the DNA was ethanol precipitated overnight at -20°C The precipitate was dissolved in TE buffer and stored as a stock of 100ng/µl.

Random Prime Labelling

REAGENTS

Hexanuclcotide mix	(Boehringer 1277 081)
Dig DNA Labelling mix	Contains 1mmol/l dATP, dCTP, dGTP,. 0.65mmol/l dTTP & 0.35mmol/l Dig-dUTP (Boehringer, 1277 065)
Klenow enzyme	2U/ml Labelling grade (Boehringer 1008 404)
Stop buffer	0.2M EDTA pH8.0
Absolute Ethanol	
TE buffer	10mM Tris pH7.5 - 8.0, 1mM EDTA

The probe was denatured by boiling 1µg of DNA for 5 min then removing to ice.The labelling reaction was carried out using reagents from the Boehringer Mannheim DIG DNA Labelling and Detection Kit.

Add to the probe	10x hexanucleotide mix	2µl
	10x dNTPlabelling mix	2µl
	sterile distilled H_2O	5µl
	Klenow enzyme	1µl
	Total volume	20µl

1. Incubate for 6hr with 2U/µl of Klenow enzyme .

2. Add 2µl of 'stop buffer'.

3. Add 2µl of 3M sodium acetate and 60µl of cold EtOH.Place in -20^{o}C freezer overnight.

4. Spin down precipitate. Decant supernantant and dry

5. Redissolved in 50µl of TE buffer to give a final stock concentration of 20 ng/µl of labelled DNA. Store in freezer.

Nick Translation Method

REAGENTS

DNase I, RNase free	Stock is 1mg/ml in 0.15M NaCl; 50% glycerol.
	(Boeringer, 776 785)

Store at -20^{o}C. Make a fresh 1:500 dilution in dH_2O for labelling.

DNA Pol 1	Stock is 10U/µl. Store at -20^{o}C.(Gibco BRL,510 80105A)
dNTPs	dATP (Pharmacia, 27-2050-01)
	dCTP (Pharmacia, 27-2060-01)
	dGTP (Pharmacia, 27-2070-01)

Stocks for nick translation reaction are at 0.5mM, prepared by a 1:200 dilution in dH_2O of 100mM stocks. Store at -20^{o}C

Dig-11-dUTP	1mM stock Store at -20^{o}C (Boehringer, 1093 088.)
Dig DNA Labelling mix	Contains 1mmol/l dATP, dCTP, dGTP, 0.65mmol/l (as before)
	dTTP & 0.35mmol/l Dig-dUTP (Boehringer, 1277 065)
	Store at -20^{o}C

TE buffer	10mM Tris pH7.5; 1mM EDTA)
Nick translation salts	0.5M Tris pH 7.5; 0.1M $MgSO_4$; 1mM dithiothreitol;
small	500µg/ml BSA fraction V (Sigma). Store at -20°C in aliquots
NICK Spin Columns	Prepacked spin columns containing Sephadex G-50 fine, DNA grade.(Pharmacia, 17-0862-01)

Label 1.0µg of DNA in each reaction mix. The DNA must be clean as nick translation is sensitive to contaminants. All components of the reaction must be kept on ice. It is particularly important to dilute the DNase in COLD dH_2O. The nucleotides may be added separately, alternatively a DIG DNA labelling mix can be used.

1. Mix in an ependorf:

	EITHER		OR
2µl	10X Nick translation salts.	2µl	10X Nick translation salts.
2.5µl	0.5mM dATP	2µl	DIG DNA labelling mix
2.5µl	0.5mM dCTP		
2.5µl	0.5mM dGTP		
2.5µl	1.0mM dig-11-dUTP		
1µl	1:500 fresh dilution of DNase (1mg/ml)	1µl	1:500 fresh dilution of DNase (1mg/ml)
xµl	probe (1µg)	xµl	probe (1µg)
yµl	dH2O	yµl	dH2O

The final volume of each = 19ml

2. Mix and spin to collect.

3. Add 1µl Pol 1.

4. Incubate for 4 hours at 15°C (in ice bucket) or overnight at 4°C.

5. Spin to collect

6. Stop the reaction by adding 2µl 0.2M EDTA and 1µl 5% SDS. Pool samples together and make up to 75-150µl with TE buffer.

After the labelling reaction the probe is separated from unincorporated nucleotides using a Sephadex spin column (Pharmacia).

1. Invert the column several times to resuspend the gel, then place it upright and allow the gel to settle.

2. Remove the top cap and then the bottom cap. Allow the column to drain. Tap the column gently to remove any air bubbles.

3. Place the column in a centrifuge tube and add 2ml of TE buffer. Allow to drain.

4. Centrifuge the tube for 4 min at 500g in a swinging bucket rotor. Remove column and discard eluate.

5. Slowly apply the sample (75-150µl) to the centre of the flat gel surface.

6. Place an uncapped eppendorf in the bottom of the tube. Place the loaded column in the centrifuge tube. Elute the sample by centrifugation for 4min at 500g.

Assume that 80% is recovered and make up to a concentration of 20ng/ml in TE buffer

IN SITU HYBRIDISATION

REAGENTS

10x PBS	Made up from tablets (Oxoid Code BR14a)
50x Denhardts solution	as before
(stored in freezer)	
20x SSC	3M NaCl, 0.3M Na_3 Citrate [pH7-7.4] Make up as before

TE buffer	10mM Tris[pH7.5], 1mM EDTA (BDH)	
Salmon sperm DNA (sonicated)	Stock is 10mg/ml in TE buffer	
		Final concentration
Prehybridisation Buffer	50x Denhardts solution	5x
	Deionised formamide	45%
	20x SSC	6x
	Salmon sperm DNA (10mg/ml)	500mg/ml
	H_20	
Hybridisation Buffer	labelled DNA	20-40ng/slide
	Salmon sperm DNA	500mg/ml
	20% Dextran Sulphate	10%
	20 x SSC	5x
	H_20	
Triton-X-100	Detergent	
Buffer 1	0.1 M Tris, 0.15M NaCl [pH7.5]	
Anti digoxigenin conjugated	polyclonal sheep anti-digoxigenin Fab fragments to HRP-antibody horseradish peroxidase.	
	150U/ml.(Boehringer-Mannheim)	
DAB buffer	0.05mM Tris [pH7.3]	
Development Reagent	0.5mg/ml diaminobenzidine tetrahydrochloride in DAB buffer Add 3µl H_2O_2 per 10ml of reagent just before use.	

METHOD

1	De-wax sections in Histoclear		2 x 10 mins at room temperature
2	Take to PBS :-	100% EtOH	2 x 5 mins
		3% H_2O_2/MeOH	30 mins (HRP ONLY)
		70% EtOH	2 x 5 mins
		PBS	2 x 5 mins
3 20	If using Alkaline phosphatase end point then treat with 20% Acetic acid for seconds then PBS for 2 x 5 mins		
4	Treat with 1mM NaOH	70°C	3 mins
5	Wash in PBS	4°C	2 x 5 mins

PREHYBRIDISATION

Make up the following mixture (enough for 10 slides);

40ul	50 x Denhardts
180ul	Deionised formamide
120ul	20 x SSC
20ul	S.Sp. DNA
40ul	H_2O

Put 35ul on a clean glass coverslip and pick up onto a slide and incubate at 60°C for 15 mins. Do not seal slides.

HYBRIDISATION

Use 40ul/slide and incubate at 60°C overnight . Use humid sandwich boxes.(2 x SSC)

Mixture	10.0ml	labelled probe*	(boil for 10 mins)
	12.5ml	Salmon.Sperm DNA	
	125.0ml	20% Dextran sulphate	
	62.5ml	20 X SSC	
	30.0ml	H_2O	

Use hydrophobic coverslips (Gelbond) to pick up mixture onto slides and seal with nail varnish.

* Probe is routinely stored at 20ng/µl .The volume used depends upon signal desired.

WASHES

1. 2 x SSC, 0.1% Triton-X	2 x 5 mins	room temperature
2. 0.1xSSC,0.1% Triton-X	1 x 5 mins	room temperature
3. 0.1xSSC,0.1% Triton-X	1 x 5 mins	50°C
4. 0.1xSSC,0.1% Triton-X,5% BSA	1 x 5 mins	room temperature

VISUALISATION METHODS

1. Wash slides in buffer 1 for 5 mins.

2. Place slides in a humidified chamber and flood with antibody

   (a) 1:2500 dilution of the anti-digoxigenin AP-antibody in buffer 1 for 30 mins.

   (b) 1:100 dilution of the anti-digoxigenin HRP-antibody in buffer 1 for 30 mins

3. Wash in buffer 1 for 2 x 10 mins (stirring)

4. Wash in buffer 3 (AP) or DAB buffer (HRP) for 5 mins (stirring)

5. Place slides in a humidified chamber and flood with development reagent for 30-60 mins (AP reaction) or 40 mins (HRP reaction).The time for the AP reaction can vary.

NB KEEP IN THE DARK

6 Stop reaction by placing slides in stop buffer (5 mins minimum) and mount with aqueous mounting medium (AP reaction). HRP slides are washed in water, dehydrated and mounted in Histomount.

If required then counterstaining can be done before mounting.

d) PROTOCOL TO DETECT β-GLOBIN TRANSGENE IN BLOOD CELLS

REAGENTS

Buffer 3	0.1M Tris	12.1g
	0.1M NaCl	5.8g
	0.05M MgCl	10.15g

make up to 1l and pHto 9.5

Take a fresh blood sample and smear onto clean slide. (10µl sufficient). Air dry.

Fix slides in Ethanol:Acetic acid (3:1) for 60 mins. Air dry.

Put slides into Acetone for 10 mins. Air dry.

Dehydrate slides through alcohols. (70%; 80%; 95%; 100% 2 mins each).

Hybridisation is done overnight at 37°C using the following mixture:

For 10 slides;	Volume	Final Conc.
Deionised Formamide	200µl	50%
20 x SSC	40µl	2 x SSC
S. Sperm DNA(10mg/ml)	40µl	10%
50% Dextran	80µl	10%
dH_2O		30µl
Probe(boiled 10 mins)	10µl	20ng/slide

Place 40µl of hybridisation mixture onto a hydrophobic coverslip (22x50mm) and pick up onto slide. Seal with nail polish and put at 75°C for 10 mins to denature material. Remove from 75_C and place in a preheated box at 37°C overnight.

WASHES

50% formamide, 2 x SSC	3 x 5 mins	45°C
2 x SSC	3 x 5 mins	45°C
4 x SSC,0.05% Triton-X	Holding Step	RT
5% Marvel,4 x SSC,0.05% Triton-X	5 mins	RT

VISUALISATION METHOD

1. Place slides in Buffer 1 for 5 mins.

2. Place slides in a humidified chamber and flood with antibody (1:2500 dilution of the anti-digoxigenin AP-antibody in buffer 1) for 30 mins

3. Wash in Buffer 1 for 2 x 10 mins

4. Wash in Buffer 3 for 5 mins

Stain	30-60 mins	Dark

Can be viewed without coverslip

IV. GEL ELECTROPHORESIS

REAGENTS

Fructose 6 phosphate (F6P)	Sigma (F3627)
Glucose 6 phosphate dehydrogenase (6-10units)(G6P)	Sigma (G8878)
Glycine	Sigma (G7126)
$MgCl_2$ $6H_2O$	BDH (10149)
NADP	Sigma (N0505)
Nitro blue tetrazolium (NBT)	Sigma (N6876)
Phenozine methosulphate (PMS)	Sigma (P9625)
Tris	Sigma(T1503)
Glycerol	BDH (28454)
Sodium citrate	BDH (10242)

Electrophoresis Buffer	0.02M Tris
	0.2M Glycine

Adjust to pH 8.1 and store for one month at $4^{o}C$.

Stock Solutions for Stain

F6P	20mg/ml
$MgCl_2$	0.2%
NADP	2.7mg/ml
NBT	2.7mg/ml
PMS	2.5mg/ml
Tris citric acid pH8	0.3M Tris
	0.08M Citric acid

Stain (1 plate)

Glycerol/MgCl2 (50:50)	1.5ml
Tris citrate	170µl
F6P	170µl
NBT	170µl
NADP	170µl
G6PD	10units
PMS	20µl

METHOD

Electrophoresis was carried out using electrophoresis tanks and Super Z-12 applicators obtained from Helena Laboratories and a Consort Bioblock Scientific power pack. Titan III celulose acetate plates (Helena Laboratories) were soaked in electrophoresis buffer for 30min. The tank reservoirs were filled with electropheresis buffer and Whatman filter paper was used to form wicks. Freeze and thaw samples three times. Sample was diluted 1 : 5 in water, applied to blotted plates and run from anode to cathode at 200V for 60min, then stained for glucose phosphate isomerase (GPI-1) activity for up to 20min

on a 37°C hotplate. After rinsing in water, the plates were fixed in 5% acetic acid for 5min then washed in distilled water for 15min.

Densitometry using using a Helena Process-24 gel scanner was carried out to determine the relative proportions of GPI-1A and GPI-1B and GPI-1C in the samples (West *et al*, 1986).

V. EMBRYO CULTURE MEDIA

STOCK SOLUTIONS

Stock A (10x)	1M NaCl
	0.05M KCl
	1.2M KH_2PO_4
	0.01M $MgSO_4.7H_2O$
	0.23M Na lactate (60% solution)
	5.5mM Glucose
	10^5 units penicillin
	750 units/mg streptomycin
Stock B (10x)	0.25M $NaHCO_3$
	0.01g phenol red
Stock C (100x)	0.33M Na pyruvate
Stock D (100x)	0.17M Ca $Cl_2.2H_20$
Stock E (10x)	0.25M HEPES (Ultrapure Calbiochem)
	0.01g phenol red

Adjusted to pH to 7.4 with 5M NaOH before making up to 100ml.

100ml of stocks A, B and E and 10ml of C and D were made. All stocks were filter sterilised using a millipore filter (0.22ml)and stored at 4^oC. Stocks A, D and E were kept for 3 months and B and C changed every two weeks.

VI. EMBRYO COLLECTION

M2 HOLDING MEDIUM

(HEPES buffered modified Kreb's-Ringer solution; Quinn *et al*, 1982)

Stock	A	1.00ml
	B	0.16ml
	C	0.10ml
	D	0.10ml
	E	0.84ml
Double distilled H_2O		7.8ml
BSA		4mg/ml

Filter sterilised before use.

Acid Tyrode's solution	0.14M NaCl
	2.6mM KCl
	1.4mM $CaCl_2$
	0.5mM $MgCl_2.6H_2O$
	0.2mM$NaH_2PO_4.H_2O$
	5.5mM Glucose
	0.12M $NaHCO_3$
	0.4% polyvinyl pyrrolidone

Adjusted to pH 2.5 with 5M HCl. Filter sterilised and stored at 4^oC.

M16 CULTURE MEDIUM

(modified Kreb's-Ringer bicarbonate solution; Whittingham, 1971)

Stock	A	1.0ml
	B	1.0ml
	C	0.1ml
	D	0.1ml
Double distilled H_2O		7.8ml
	BSA	4mg/ml

Filter sterilised before use.

Preimplantation embryos were removed from the uterus and oviducts by inserting a syringe needle into one end of the uterus (or oviduct) and flushing M2 medium through. The embryos were then collected and placed in a fresh drop of M2. To remove the zona pellucidae, groups of approximately 6 embryos were placed in a watchglass previously heated to 37^{o}C containing acid Tyrodes solution and watched closely until the zona begins to disappear. This occurred quickly in a matter of seconds. The embryos were then rinsed in a fresh drop of M2 to remove any traces of the acid Tyrode solution [pH2.5]. Once the zona has been removed the embryos become 'sticky' and are more difficult to handle. Chimaeras were produced by aggregating pairs of 8-cell embryos together To aid fusion, the 2 embryos are placed together in a drop a of M2 + PHA (1 part phytohaemagglutinin [M form, GIBCO 670-0576] plus 19 parts M2 medium) and pushed together, they were left for 2-3mins to ensure that they have stuck and are washed in a fresh drop of M2. The embryos are cultured overnight in drops of M16 culture medium under oil which has been allowed to equilibrate at 37^{o}C in 5% CO_2. The next day the aggregated embryos had reached blastocyst stage in development, those embryos that had not aggregated properly are discarded. The blastocysts were then transferred to pseudopregnant females.

VII. SUPEROVULATION

PMSG Stock solution of 50units/ml. Dosage : 5IU per mouse

hCG Stock solution of 50units/ml. Dosage : 5IU per mouse

Females to be superovulated were injected intraperitoneally with 0.1ml pregnant mares' serum gonadotrophin (PMSG) at 12 noon followed 48hrs later by injection of 0.1ml human Chorionic gonadotrophin (hCG). Females were then mated to males of the required strain and vaginal plugs checked the next morning. The day of plug detection was designated $^1/_2$ day *post coitum* (*p.c.*). Pregnant females were killed by cervical dislocation on day $2^1/_2$ and the oviducts and uterus were removed. On the day of detection of vaginal plug, a group of CF1 females was examined and those in oestrus were mated to vasectomised CF1 males to provide pseudopregnant females

VIII. TRANSFER OF EMBRYOS

REAGENTS

Anaesthetic

Hypnorm (0.315 mg/ml fentanyl citrate and 10mg/ml fluanisone; Janssen Pharmacuticals)

Hypnovel (2mg/ml midazlam hydrochloride; Roche)

Females to be used as transfer recipients were selected in oestrus and mated to vasectomized males. Vaginal plugs were checked the following morning, the day of detection was designated as day $^1/_2$. Transfer took place on day $2^1/_2$. Pseudopregnant females were anaesthetised with 0.25 ml per 30 g body weight of a 1:1 v/v mixture of Hypnorm and Hypnovel. Both the Hypnorm and Hypnovel were diluted 1:1 v/v with sterile distilled water, before being mixed together. Pregnancies were timed according to the pseudopregnant female.

The embryos were collected in a small amount of M2 handling medium in a fine glass pipette, and were surgically transfered to the uterus, as close to the oviduct as possible, via a small incision made with a fine needle (McLaren and Michie, 1956).

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